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

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The majority of hepatic intracellular triacylglycerol (TG) is mobilized by lipolysis followed by reesterification to reassemble TG before incorporation into a very-low-density lipoprotein (VLDL) particle. Triacylglycerol hydrolase (TGH) is a lipase that hydrolyzes TG within hepatocytes. Immunogold electron microscopy in transfected cells revealed a disparate distribution of this enzyme within the endoplasmic reticulum (ER), with particularly intense localization in regions surrounding mitochondria. TGH is localized to the lumen of the ER by the C-terminal tetrapeptide sequence HIEL functioning as an ER retention signal. Deletion of HIEL resulted in secretion of catalytically active TGH. Mutation of HIEL to KDEL, which is the consensus ER retrieval sequence in animal cells, also resulted in ER retention and conservation of lipolytic activity. However, KDEL-TGH was not as efficient at mobilizing lipids for VLDL secretion and exhibited an altered distribution within the ER. TGH is a glycoprotein, but glycosylation is not required for catalytic activity. TGH does not hydrolyze apolipoprotein B-associated lipids. This suggests a mechanism for vectored movement of TGs onto developing VLDL in the ER as TGH may mobilize TG for VLDL assembly, but will not access this lipid once it is associated with VLDL.

INTRODUCTION

Hepatic very-low-density lipoprotein (VLDL) assembly is an intricate process that is largely regulated by the provision of lipid (Borchardt and Davis, 1987; Dixon *et al.*, 1991; White *et al.*, 1992). When provision of neural lipid is inadequate or limiting, the nascent lipoprotein particle is degraded intracellularly (Pullinger *et al.*, 1989; Dixon *et al.*, 1991; White *et al.*, 1992 and reviewed in Yao *et al.*, 1997; Fisher and Ginsberg, 2002). Hence mobilization of stored lipid represents a potentially regulated step in VLDL production and secretion. Triacylglycerols (TGs) represent the largest constituent of VLDL lipids (Davis and Vance, 1996). Several groups using different approaches have shown that the bulk of

VLDL-TG (~70%) is derived from intracellular stores that must undergo lipolysis, followed by reesterification of the lipolytic products to reform TGs before incorporation into a VLDL particle (Wiggins and Gibbons, 1992; Yang *et al.*, 1996; Lankester *et al.*, 1998). The enzyme triacylglycerol hydrolase (TGH), initially purified from porcine liver microsomes, has been suggested to play a role in this process (Lehner and Verger, 1997). We cloned the human TGH cDNA (Alam *et al.*, 2002a; GenBank accession no. NM_001266) and identified the amino acid residues involved in a catalytic triad as well as a glycosylation site (Alam *et al.*, 2002b). TGH is a 60-kDa soluble protein localized to the lumen of the endoplasmic reticulum (ER) and is a member of the carboxylesterase family of enzymes (EC 3.1.1.1). Hepatoma cell lines lacking this enzyme (e.g., McArdle RH7777 cells and HepG2) are inefficient in the mobilization of stored TG for VLDL secretion (Gibbons *et al.*, 1994; Wu *et al.*, 1996; Lehner and Vance, 1999). Expression of TGH in hepatoma cells increased mobilization of intracellular TG and lipidation of the primary protein component of VLDL, apolipoprotein (apo) B100 (Lehner and Vance, 1999). Inhibition of intracellular lipolysis by specific chemical inhibitors of TGH decreased apoB and TG secretion in both rat hepatocytes and in hepatoma cells expressing TGH (Gilham *et al.*, 2003). Collectively these data demonstrate the involvement of TGH in VLDL assembly (Dolinsky *et al.*, 2004a; Gilham and Lehner, 2004).

TGH distribution within the ER may be critical for its efficient participation in VLDL assembly because it could dictate proximity to substrate pools or apoB. Juxtaposition to

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Abbreviations used: Apo, apolipoprotein; BiP, ER-binding protein; CE, cholesteryl ester; ER, endoplasmic reticulum; HS, horse serum; HSL, hormone-sensitive lipase; LPL, microbial lipoprotein lipase; MUH, 4-methylumbelliferyl heptanoate; PBS, phosphate-buffered saline; TG, triacylglycerol; TGH, triacylglycerol hydrolase; U, enzymatic unit; VLDL, very-low-density lipoprotein.

these entities could impact the ability of TGH to direct lipids toward assembly of nascent VLDL. We investigated whether the localization of TGH within the ER is influenced by a C-terminal sequence believed to retain TGH within this compartment and if secretion of neutral lipid in transfected cells is affected by mutation of this sequence. We also characterized the requirement of glycosylation for catalytic activity as glycosylation is known to mediate interaction with chaperones found in the ER that facilitate protein folding. In addition, we examined whether neutral lipid on apoB-containing particles could be included in the TGH substrate pool as they coexist in the ER lumen.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized by the Institute for Biomolecular Design at the University of Alberta. DNA sequencing was performed by the Molecular Biology Services Unit at the University of Alberta. The plasmid pCI-neo was purchased from Promega (Madison, WI). QuikChange Site-Directed Mutagenesis Kits and monoclonal antibody (mAb) specific for the flag epitope were from Stratagene (La Jolla, CA).

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), horse serum (HS), Geneticin, Lipofectamine2000, TRIZOL Reagent, Superscript II reverse transcriptase, and the plasmid pBudCE4.1 were purchased from Invitrogen Canada (Burlington, Ontario, Canada).

4-Methylumbelliferyl heptanoate (MUH), *p*-nitrophenyl laurate, tunicamycin, bovine serum albumin (BSA), paraformaldehyde, *tert*-butyl hydroperoxide, and NADH as well as rabbit polyclonal antibodies for the myc epitope and 10 nm-gold conjugated anti-rabbit IgG were from Sigma-Aldrich (Oakville, Ontario, Canada).

Polyclonal antibodies for TATA-binding protein were from Santa Cruz Biotechnology (Santa Cruz, CA). mAb for the myc epitope was obtained from Clontech (Palo Alto, CA), and polyclonal antibodies for calnexin and mAb for BiP (also known as Grp78) were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Polyclonal anti-apoB antibodies were purchased from Chemicon International (Temecula, CA). Prolong Antifade and fluorescently labeled secondary antibodies were purchased from Molecular Probes (Eugene, OR). IRDye 800-conjugated anti-rabbit IgG secondary antibodies were from Rockland Immunochemicals (Gilbertsville, PA).

[9,10(*n*)-³H]Oleic acid (10Ci/mmol) and immunoblotting reagents were obtained from Amersham-Pharmacia Canada (Oakville, Ontario, Canada). [9,10(*n*)-³H]triolein was purchased from New England Nuclear (Boston, MA). The TGH specific inhibitor (4,4,4-trifluoro-2-[2-(3-methylphenyl)hydrazono]-1-(2-thienyl)butane-1,3-dione) was from GlaxoSmithKline (Les Ulis, France). Anti-ADRP antibodies were a kind gift from Dr. Constantine Londos (NIH, Bethesda, MD). All other chemicals and reagents were acquired from local suppliers and were of the highest quality available.

Cell Culture

McArdle RH7777 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS (vol/vol) and 10% HS. Cos-7 cells, also from ATCC, were cultured in DMEM containing 10% FBS. All incubations were performed at 37°C in an atmosphere enriched with 5% CO₂ and in the presence of antibiotics.

Lipase Assay

Lipase activity was assayed using two methods. A fluorescence-based assay using MUH as a substrate was performed essentially as described previously (Dolinsky *et al.*, 2004b). Alternately, lipase activity was monitored spectrophotometrically at 405 nm via the liberation of *p*-nitrophenol from *p*-nitrophenyl laurate as previously described (Lehner and Verger, 1997), except that the assays were performed in 96-well clear microtiter plates and read on a Molecular Devices SpectraMax 250 (Sunnyvale, CA). The specific method used is indicated in figure legends.

Production of ΔR-TGH and KDEL-TGH Mutants

A deletion mutant of TGH that lacks the coding region for the C-terminal four amino acids (–HIEL) was generated by PCR using the forward primer: 5'-tactgtcagctctcagatgttgctcgtgctcttctac-3' and the reverse primer: 5'-tgacgttagctgtgggtacctctctgtctggtgggtgctctc-3' and the full-length TGH cDNA (Alam *et al.*, 2002a) as a template. The PCR product was cloned into the pCI-neo mammalian expression vector and termed ΔR-TGH.

A TGH mutant bearing the amino acid sequence –KDEL at its C-terminus, rather than –HIEL, was generated exactly as ΔR-TGH, except using the reverse primer: 5'-tgacgttagctgtgggtacctcagctcatctcttctgtctggtgggtgctt-3'. The

cDNAs were sequenced to ensure integrity and used to stably transfect McArdle RH7777 cells.

Stable Transfection of McArdle RH7777 Cells

In a 60-mm-diameter dish, 1.6×10^6 McArdle RH7777 cells were plated and grown overnight. Three micrograms of plasmid were introduced using Lipofectamine2000 according to the manufacturer's instructions. Cells were grown for 24 h after transfection and then passaged 1:10 into media containing 1.6 mg/ml Geneticin. Single colonies were selected and characterized.

RT-PCR of ΔR-TGH

RNA from 60-mm-diameter dishes of 80% confluent ΔR-TGH-expressing McArdle RH7777 cells was isolated using TRIZOL Reagent according to the manufacturer's instructions. Total RNA was reverse transcribed using oligo dT₁₈ primer and Superscript II reverse transcriptase. A 1-kb region of ΔR-TGH was amplified using the following primers: forward: 5'-gcattctggggattcttcagcagggat-gaacacagcgc-3' reverse: 5'-gagcaagttggccagatttcatcaccatttgcag-3'. Selected amplicons were purified and sequenced. Cyclophilin was amplified as previously described (Agellon *et al.*, 2002).

Characterization of ΔR-TGH

In 60-mm-diameter dishes, 1.5×10^6 cells each of untransfected McArdle RH7777 cells and McArdle RH7777 cells stably transfected with *wt*-TGH, ΔR-TGH or empty pCI-neo were allowed to settle and attach to the dishes overnight. Cells were then incubated 6 h in 2 ml serum-free DMEM. The media were collected and centrifuged at $600 \times g$ for 2 min to remove cell debris. Cells were scraped into buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose. The cell suspensions were briefly sonicated and microsomes were prepared from postmitochondrial supernatants (Lehner and Kuksis, 1992). Microsomes were resuspended in 1 ml of phosphate-buffered saline (PBS) containing Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN). Seventy microliters of media and 35 μL of resuspended microsomes were electrophoresed in denaturing SDS-PAGE (10%) and then transferred to a nitrocellulose membrane. TGH and albumin were probed by immunoblotting with antihuman TGH (Alam *et al.*, 2002a) and anti-rat albumin (Alam *et al.*, 2002a; Gilham *et al.*, 2003) polyclonal antibodies generated in our laboratory.

Tunicamycin Treatment

In 100-mm-diameter dishes, 4.5×10^6 McArdle RH7777 cells stably expressing ΔR-TGH were grown overnight. Cells were subsequently incubated for 1 h in DMEM containing 10% FBS, 10% HS, and $\pm 5 \mu\text{g/ml}$ tunicamycin. Cells were washed with PBS and incubated for 5 h in serum free DMEM $\pm 5 \mu\text{g/ml}$ tunicamycin. Media (7 ml) were collected and centrifuged at $600 \times g$ for 2 min to pellet cell debris. Cells were scraped into ice-cold PBS and sonicated briefly. The amount of protein in these lysates was determined. Media were concentrated 10-fold using a Millipore Ultrafree centrifugal filter device (Bedford, MA) with a semipermeable membrane with a molecular weight exclusion of 10 kDa. Protein in aliquots of concentrated media proportional to the amount of cellular protein were separated by SDS-PAGE (10%) and transferred to nitrocellulose. The amounts of TGH and albumin were assessed by immunoblotting using respective polyclonal antibodies as above.

Production and Analysis of a Nonglycosylated Secreted TGH Mutant

The lone glycosylation site in TGH was disrupted by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions. The template for mutagenesis was ΔR-TGH in pCI-neo. Primer sequences were as follows: forward: 5'-gctttgtgaagcaagcacctcgtacc-3' reverse: 5'-ggtacgaggtgctgcttcacaaagc-3'. The result was a construct coding for a nonglycosylated, secreted mutant (N79QΔR-TGH).

The N79QΔR-TGH construct was stably transfected into McArdle RH7777 cells. Cells from a resulting cell line and untransfected cells were plated on 60-mm dishes and incubated overnight. Media were changed to 2 ml of serum-free DMEM and incubated for an additional 12 h. Media were cleared of cell debris by centrifugation and lipase activity in 100 μL was measured. To determine if activity was due to TGH in the media, a TGH-specific inhibitor was included in concurrent assays at a final concentration of 10 μM. Cells were scraped into PBS and briefly sonicated. Fifty micrograms of cellular protein and 65 μL of media were subjected to denaturing SDS-PAGE (10%). Immunoblots were performed to detect TGH protein as above.

Access of KDEL-TGH versus *wt*-TGH to Endogenous Substrate

Untransfected McArdle RH7777 cells (1.6×10^6) or cells stably transfected with either *wt*-TGH or KDEL-TGH were plated on 60-mm diameter dishes and grown overnight. These cells were subsequently incubated in DMEM containing 0.4 mM oleate complexed to 0.5% essentially fatty acid-free BSA for 2.5 h to promote TG deposition. [³H]Oleate at 5 μCi/dish was included as a tracer. Cells were washed three times for 10 min with DMEM containing

0.5% BSA to remove labeled fatty acids that were nonspecifically associated with the exterior of the cells. Cells were then incubated 4 h in 2 ml of DMEM. Media were collected and cleared of cell debris by centrifugation at $1700 \times g$ for 2 min. Cells were harvested in 2 ml of PBS and sonicated. Lipids from 1-ml aliquots of cells and media were extracted in 4 ml chloroform:methanol (2:1; Folch *et al.*, 1957) containing nonradiolabeled lipid carriers. Extracted lipids were resolved by TLC and the associated radioactivities were measured by scintillation counting as previously described (Lehner and Vance, 1999). A lipase assay was performed on 4 μ g of protein from cell sonicates. Sixty micrograms of protein were used in SDS-PAGE (10%), and immunoblots were performed using anti-TATA-binding protein polyclonal antibodies according to manufacturer's instructions, and for TGH as above.

Specific Enzyme Activity of TGH and TGH Mutants

To determine if mutations to TGH engineered during this investigation affected enzymatic activity, we performed *in vitro* lipase assays on cell lysates with normalized amounts of TGH. McArdle RH7777 cells stably expressing *wt*-TGH, a mutant of TGH or stably transfected with empty plasmid were grown to ~80% confluence on 60-mm dishes. Cells were scraped into PBS and lysed by brief sonication, and protein concentration was determined as above. SDS-PAGE was used to separate 45 μ g of protein from each cell lysate. Proteins were transferred to a nitrocellulose membrane. Immunoblots were performed using anti-TGH polyclonal antibodies as above and IRDye 800-conjugated anti-rabbit IgG secondary antibodies according to the manufacturer's instructions. Fluorescence intensity for the TGH band in each lane was quantitated using a Li-Cor Odyssey Infrared Imaging System (Lincoln, NE). Cell lysate containing equal amounts of TGH (*wt*-TGH or mutants) were used in lipase activity assays.

Electron Microscopy of TGH in McArdle RH7777 Cells

The subcellular localization of TGH was assessed by immunogold electron microscopy. The ultrathin frozen sections of *wild-type* and TGH-transfected cells were obtained and processed essentially as described by Cui *et al.* (1993). Ultrathin cryosections were incubated with affinity-purified anti-TGH polyclonal antibodies. Primary antibodies were revealed by incubation for 1 h with colloidal gold 10-nm gold-conjugated anti-rabbit IgG. Sections were subsequently stained with 2% uranyl acetate for 30 min and lead citrate for 5 min and examined in a Hitachi H-7000 electron microscope (Pleasanton, CA).

Production of myc-tagged KDEL-TGH and flag-tagged *wt*-TGH

Sequences for myc and flag epitope tags were introduced to the *wt*-TGH cDNA by PCR using the common forward primer 5'-cggaattcatgtgctcgtgcttattacc-3'. Reverse primers were 5'-gctggatcttcattagatcacagctctatgtgctatcgtctatcctgtaattctctgtcgggtgcttctcactgc-3' to introduce the flag sequence immediately upstream of the region coding for -HIEL and 5'-gctggatcttcattagatcacagctctatgtcaggtctctctctgagatcagctctctctctgtcgggtgcttctcactgc-3' to incorporate the myc-tag. These PCR products were ligated into pCI-neo. The region coding for the C-terminus of the myc-tagged construct was then mutated to myc-KDEL via site directed mutagenesis. The primers used were as follows: forward: 5'-ggaggactgaaagacgagctgtgaagatctgtcgaccggg-3' and reverse: 5'-ccgggtcgacagatcttcacagctgtcttctcaggtctctcc-3'. The cDNAs were sequenced to ensure unwanted errors were not introduced during PCR or mutagenesis.

The pBudCE4.1 vector has two promoters and two cloning sites. The myc-tagged KDEL-TGH and flag-tagged *wt*-TGH constructs were excised from pCI-neo using restriction endonucleases, purified from an agarose gel, and ligated into pBudCE4.1. This plasmid containing both TGH constructs, as well as empty pBudCE4.1 for control experiments, were used to stably transfect McArdle RH7777 cells.

Colocalization of *wt*-TGH and KDEL-TGH with Each Other and with Neutral Lipid Droplets

Transfected McArdle RH7777 cells were grown on sterile coverslips overnight in 6-well plates (2×10^5 cells/well). Cells were cultured in serum-free medium for 1 h to facilitate removal of immunoglobulins before staining and then fixed to coverslips with 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 for 5 min and incubated with the indicated primary antibodies diluted 1:100–1:1000 with 3% BSA in PBS for 1 h. After washing, cells were incubated with fluorescently labeled secondary antibodies (either fluorescein, Alexa488, or Texas Red) directed at the appropriate IgG species for 1 h. Coverslips were mounted on microscope slides with Prolong Antifade. When observing colocalization with neutral lipid droplets, cells were incubated with 0.4 mM oleate complexed to 0.5% fatty acid-free BSA for 4 h before fixing them to cover-slips. Neutral lipid droplets were stained with Nile Red diluted 1:1000 for 10 min after antibody staining had been completed. Images were produced using a Zeiss LSM510 confocal microscope (Thornwood, NY) with an argon laser delivering a wavelength of 488 nm to excite fluorescein or Alexa488 and a HeNe laser delivering 543 nm to excite Texas Red or Nile Red.

Density Gradient Ultracentrifugation of *wt*- and KDEL-TGH

McArdle RH7777 cells stably expressing both myc-tagged KDEL-TGH and flag-tagged *wt*-TGH were grown to ~80% confluence on a 100-mm dish and then incubated in DMEM containing oleic acid complexed to BSA for 4 h. Cells were washed with PBS and then scraped into 1.8 ml of 2 mM Tris, pH 8.8, containing Complete protease inhibitors. Cells were homogenized using a Potter Elvehjem apparatus. Homogenates were centrifuged at $500 \times g$ for 10 min at 4°C. An 800- μ l aliquot was adjusted with an equal volume of glycerol and transferred to a Beckman Quick-Seal centrifuge tube (Fullerton, CA). The samples were overlaid with 1.5 ml of buffer containing 250 mM sucrose, 1 mM EDTA, and 50 mM Tris, pH 7.4, and an additional layer containing 0.9% NaCl. The tube was centrifuged at 60,000 rpm in a Beckman VTi65.2 rotor for 45 min at 4°C. Fractions of 0.5 ml were collected from the bottom of the tube. Fifty-microliter aliquots of each fraction were analyzed by SDS-PAGE followed by immunoblotting using antibodies directed against the myc epitope, flag epitope, calnexin, and the lipid droplet coat protein adipose differentiation-related protein (ADRP; Londos *et al.*, 1999). Immunoblots were performed in accordance with protocols provided by the suppliers. Relative intensities of the bands for the myc and flag epitopes were determined by densitometry using Bio-Rad Quantity One software (Hercules, CA).

Hydrolysis of [3 H]oleate-labeled Lipoproteins by TGH

McArdle RH7777 cells were grown to ~80% confluence in 60-mm diameter dishes. Media were changed to 2 ml of DMEM containing 0.4 mM oleate, 0.5% BSA and 5 μ Ci [3 H]oleate for 2.5 h. After this loading period, cells were washed three times for 10 min in DMEM containing 0.5% BSA to remove unincorporated fatty acids. The cells were then incubated 4 h in DMEM. Aliquots of this medium containing labeled lipoproteins were transferred onto McArdle RH7777 cells stably transfected with Δ R-TGH, or nontransfected cells $\pm$ 1.2 units (U) of lipoprotein lipase (LPL) from *Chromobacterium viscosum* (Sigma, St. Louis, MO). (One unit is defined as the amount of enzyme that liberates 1 μ mol of oleate from triolein per min at pH 8.0 and 40°C.) One milliliter of media from each incubation was collected after 14 h and lipids were extracted, separated by TLC, and analyzed by scintillation counting as above. Aliquots of media from dishes of Δ R-TGH or nontransfected cells $\pm$ 0.6, 1.2, and 2.5 U LPL were also analyzed for lipase activity.

To assess intracellular apoB-containing particles as a substrate for TGH, hepatocytes were isolated by collagenase perfusion of the liver from male Sprague Dawley rats (body wt ~150 g) fed ad libitum essentially as previously described (Yao and Vance, 1988). Hepatocytes were plated on 100-mm collagen-coated cell culture dishes in DMEM containing 15% FBS and allowed to settle and attach to the dishes for 5 h. Media were changed and incubation continued for 14 h. Media was then replaced with DMEM containing 0.4 mM oleate, 0.5% BSA, and 8 μ Ci/ml [3 H]oleate for 4 h. Cells were washed twice with PBS and scraped into buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 250 mM sucrose. Cells were sonicated briefly and then microsomes prepared as above. Microsomes were resuspended in 0.2 M sodium carbonate, pH 12, and placed on ice for 15 min. The suspension was then centrifuged at $350,000 \times g$ for 45 min at 4°C. The supernatants, containing microsomal luminal contents, were transferred to new tubes, and the pH was adjusted with 1 M Tris-HCl, pH 7.4. ApoB-containing particles were isolated from the supernatants by immunoprecipitation with anti-apoB antibodies bound to protein A-Sepharose beads. Beads carrying apoB-containing particles were washed twice with PBS and then divided into 10 equal aliquots. One aliquot was placed at 4°C until analysis of all other samples. Three aliquots received DMEM that did not contain lipases, three received DMEM containing Δ R-TGH, and three received an equal volume of DMEM containing LPL with the same *in vitro* lipolytic activity with MUH as those with Δ R-TGH. Samples were incubated for 14 h at 37°C while rotating end-over-end. All samples were then treated with chloroform:methanol 2:1, lipids were extracted, separated via TLC and radioactivity associated with different lipid species was determined by scintillation counting as above.

Assessing Cell Integrity by Lactate Dehydrogenase Activity Assays

Lactate dehydrogenase (LDH) activity in both cell lysates and media of McArdle RH7777 cells was performed essentially as previously described (Moldeus *et al.*, 1978; Haidara *et al.*, 2002; Gilham *et al.*, 2003). Briefly, McArdle RH7777 cells (untransfected or Δ R-TGH expressing) were plated on 60-mm dishes and incubated overnight. Media were removed and replaced with serum-free DMEM $\pm$ 1.2 or 2.5 U LPL for 14 h. Some cells were incubated for 2 h with 2 ml of DMEM containing 400 μ M *tert*-butyl hydroperoxide. This treatment results in disruption of cell integrity and release of the normally cytosolic LDH into the medium. Samples of media were collected after incubation periods and cleared of cellular debris by centrifugation at $2600 \times g$ for 2 min. Cells were scraped into 1 ml of PBS containing Complete protease inhibitors and briefly sonicated. Assays were conducted in triplicate in clear 96-well microtiter plates with 15 μ L of culture medium or 7.5 μ L of cell sonicates supplemented with 7.5 μ L of PBS. The assay was initiated by addition of 250 μ L of substrate solution containing 100 mM phosphate buffer,

pH 7.4, 1.4 mM sodium pyruvate, and 0.2 mM NADH. Absorbance at 340 nm was then monitored every minute for 10 min. LDH activity is expressed the percent of LDH activity in the media relative to total (cells plus media) after 3 min of reaction time.

RESULTS

A Carboxy-terminal Tetrapeptide Is Responsible for Microsomal Retention of TGH

To assess the mechanism of ER retention for TGH, we constructed a deletion mutant that lacks the coding region for the C-terminal four amino acids HIEL (Δ R-TGH) and expressed the construct in McArdle RH7777 cells, a cell line that does not endogenously express TGH (Lehner and Vance, 1999). After stable transfection, a RT-PCR product could be detected in several of the resulting cell lines using TGH specific primers (Figure 1A). Clones 3 (Δ R3-TGH) and 12 (Δ R12-TGH) were analyzed further. An immunoreactive species was detected in the media and in isolated microsomes from cell lines developed from both of these colonies (Figure 1B). Cells transfected with *wt*-TGH contained TGH in microsomes, but did not secrete the protein into the media, indicating that the C-terminal 4 amino acid sequence was responsible for microsomal retention (Figure 1B). A dramatic increase in lipase activity was also seen in the media from Δ R3-TGH- and Δ R12-TGH-expressing cells compared with cells transfected with *wt*-TGH, the empty plasmid, or to untransfected cells (Figure 1C). Collectively these data demonstrate that Δ R-TGH can be secreted as a functional enzyme and also indicate that the microsomal environment is not required for enzymatic activity.

Glycosylation Is Not Required for Enzymatic Activity

TGH is a glycoprotein (Alam *et al.*, 2002a) with a single N-glycosylation site at asparagine 79. To investigate the requirement of glycosylation for catalytic activity of TGH, we treated Δ R-TGH-expressing cells with tunicamycin, a potent inhibitor of N-glycosylation. Immunoblots performed on media samples from Δ R-TGH-expressing cells treated with tunicamycin showed 2 bands for Δ R-TGH, representing the glycosylated and nonglycosylated enzyme (Figure 2A). Without tunicamycin, only fully glycosylated TGH is secreted (Figure 2A). The amount of albumin in the media did not change with tunicamycin treatment, indicating the production of proteins and their movement through the secretory pathway was not appreciably altered. Despite the substantial reduction in glycosylation of Δ R-TGH with tunicamycin treatment (>60%), there was no decrease in TGH activity in the media compared with untreated Δ R-TGH-expressing cells (Figure 2B). These data demonstrate that glycosylation is not required for enzymatic activity of TGH and also suggest that correct folding of the protein during synthesis is maintained.

To further define a potential requirement of glycosylation for TGH, we created a secreted mutant in which the glycosylation site had been disrupted (N79Q Δ R-TGH). The mutation of asparagine to glutamine is conservative, maintaining the charge on this amino acid residue while preventing glycosylation. The construct was used for stable transfection of McArdle RH7777 cells. The immunoblot in Figure 3A shows the N79Q Δ R-TGH protein was synthesized and secreted. The transfected cells exhibited approximately twofold increase in lipase activity in media over samples from untransfected cells (Figure 3B). To show that the additional activity was due to the presence of N79Q Δ R-TGH in the media, we included a TGH-specific inhibitor in the assay (Gilham *et al.*, 2003). The inhibitor reduced the relative lipase activity to the same levels observed with media from untransfected cells. Similar results were derived

Figure 1. A carboxy-terminal sequence is responsible for retention of triacylglycerol hydrolase (TGH) within the ER. (A) The cDNA for human TGH lacking the coding region for the carboxy-terminal four amino acids (Δ R-TGH) was stably transfected in McArdle RH7777 cells. RT-PCR was used to detect the endogenous cyclophilin (loading control) or transfected TGH mRNA from resulting cell lines (30 cycles). (B) Immunoblots using anti-TGH and anti-albumin polyclonal antibodies to probe microsomes or media from McArdle RH7777 cells stably expressing Δ R-TGH, *wt*-TGH, or untransfected cells (McA) after an overnight incubation. (C) Lipase activity in media from McArdle RH7777 cells stably expressing *wt*-TGH, Δ R-TGH, transfected with empty plasmid or untransfected cells after an overnight incubation (*p*-nitrophenyl laurate as substrate). Data represent liberation of *p*-nitrophenol measured spectrophotometrically. Shown are the mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

from Cos-7 cells transiently transfected with the N79Q Δ R-TGH construct (unpublished data). Immunoblots employing fluorescent secondary antibodies were used to quantitate the amount of TGH in lysates from cells stably producing *wt*-TGH, Δ R-TGH, or N79Q Δ R-TGH. Lipase assays with normalized amounts of TGH protein show that specific enzyme activity is not substantially altered by removal of the C-terminal retention sequence or mutation of the glycosylation site (Figure 3C). These studies confirm that glycosylation of TGH is not a required modification for catalytic activity.

KDEL-TGH Is Less Effective in Mobilizing Neutral Lipids for VLDL Secretion than *wt*-TGH

TGH cofractionates with ER elements and lipid droplets (Lehner *et al.*, 1999). We further defined its location by immunogold electron microscopy. The electron micrograph

Figure 2. Tunicamycin decreases glycosylation of ΔR -TGH, but does not reduce its activity. (A) McArdle RH7777 cells stably expressing ΔR -TGH were treated with tunicamycin as described in *Materials and Methods*. Relative amounts of both ΔR -TGH and albumin were assessed in 10-fold concentrated media by immunoblotting using respective polyclonal antibodies. (B) Lipase activity in 10-fold concentrated media of tunicamycin-treated McArdle RH7777 cells (McA) and McArdle RH7777 cells expressing ΔR -TGH were assessed using *p*-nitrophenyl laurate as substrate. Data are the mean $\pm$ SD of triplicate samples and are representative of three independent experiments.

in Figure 4 was produced by analysis of McArdle RH7777 cells stably transfected with *wt*-TGH. It illustrates that TGH is not evenly distributed throughout the ER, but rather is found in cisternae in proximity to mitochondria. Because of this nonuniform distribution of TGH in the ER, we speculated that TGH may be targeted to a subdomain of this organelle that is specialized in VLDL production or has better ability to channel lipids toward VLDL assembly and secretion. Further, we hypothesized that the C-terminal sequence on TGH (–HIEL) may be responsible for directing the protein to a distinct region within this compartment such as mitochondria-associated membranes (MAMs; Vance, 1990; Rusiñol *et al.*, 1994). MAMs coisolate with mitochondria, but can be separated by density gradient centrifugation. MAMs are enriched in lipid biosynthetic enzyme activities compared with the bulk of ER, and several lipid-metabolizing enzymes have been shown to be enriched in these membranes, including acyl-CoA synthetase 4 (peripheral; Lewin *et al.*, 2002) and phosphatidylethanolamine *N*-methyltransferase (transmembrane; Cui *et al.*, 1993). To investigate whether the C-terminal –HIEL sequence allows better access to lipid substrates, we created a mutant of TGH bearing the consensus for ER retention in animal cells at the extreme C-terminus, i.e., KDEL-COOH and generated McArdle RH7777 cells lines stably expressing this mutant. The immunoblot in Figure 5A indicates that the KDEL-TGH-transfected cells used in these experiments express more TGH protein than cells expressing *wt*-TGH. In vitro lipase activity measurements also showed that the amount of activity in cell sonicates from KDEL-TGH-expressing cells was higher than that from *wt*-TGH-expressing cells

Figure 3. A nonglycosylated, secreted mutant of TGH is produced in an active state from transfected McArdle RH7777 cells. (A) McArdle RH7777 cells were stably transfected to produce a secreted glycosylation mutant of TGH (N79Q ΔR -TGH). N79Q ΔR -TGH protein was detected in the media after an overnight incubation via immunoblot using anti-TGH polyclonal antibodies. (B) Lipase activity in media of N79Q ΔR -TGH stably transfected McArdle RH7777 cells were determined using 4-methylumbelliferyl heptanoate (4-MUH) as substrate. A TGH-specific inhibitor was included in the assay to resolve whether increases in lipase activity were legitimately due to the production of TGH. Data characterize activity found in media via liberation of 4-methylumbelliferone. Shown are the mean $\pm$ SD of triplicate samples and are representative of three independent experiments. (C) The quantity of *wt*-TGH or mutants of TGH in cell lysates from stably transfected McArdle RH7777 cells were normalized via immunoblots using fluorescent secondary antibodies. Lysates containing equal amounts of TGH were then used in an in vitro lipase activity assay with 4-methylumbelliferyl heptanoate (4-MUH) as substrate. Data are the mean $\pm$ SD of three samples.

(Figure 5B). The enzymatic activity of KDEL-TGH appears to be similar to that of *wt*-TGH because the ratio of KDEL versus *wt*-TGH expression in transfected McArdle RH7777 cells was calculated to be 4:1 by densitometric analysis of Figure 5A, which agrees well with the observed 4:1 ratio in lipase activity (Figure 5B). To confirm this observation, the amount of *wt*-TGH and KDEL-TGH in cell sonicates was

Figure 4. Immunogold labeling and electron microscopy of transfected TGH in McArdle RH7777 cells. Immunogold labeling of TGH was performed as described in *Materials and Methods*. Magnification, $\times 97,000$. Arrows point to ER elements containing immunogold labeled TGH. N, nucleus; M, mitochondria; G, Golgi.

quantitated in immunoblots with fluorescent secondary antibodies as described above. Lipase assays using normalized amounts of *wt*-TGH or KDEL-TGH protein show that the mutation did not reduce the specific *in vitro* catalytic activity of the enzyme (Figure 5C); in fact, a slight increase was observed with KDEL-TGH.

McArdle RH7777 cells transfected with *wt*-TGH secrete more robustly lipidated VLDL particles (Lehner and Vance, 1999). We assayed the ability of KDEL-TGH to mobilize and secrete intracellular lipids as VLDL and observed that *wt*-TGH-expressing cells are able to secrete more preformed labeled TG and cholesteryl ester (CE) than KDEL-TGH-expressing cells during a chase period (Figure 5, D–F). The amount of lipid synthesis did not differ among the cell lines (unpublished data). Hence even with higher levels of intracellular TGH protein and higher cellular *in vitro* lipase activity, the KDEL-TGH-expressing cells secreted less preformed lipids than *wt*-TGH-expressing cells. Results were confirmed in a third KDEL-TGH-expressing cell line (unpublished data). These results suggest that the C-terminal sequence confers differential access to substrates and may be related to an altered distribution of KDEL-TGH within the ER lumen, however, immunogold electron microscopy of KDEL-TGH in transfected cells showed that the mutant was also localized to the lumen of the ER and some of the staining was also observed in relative proximity to mitochondria (Figure 5G).

Subcellular Localization of *wt*-TGH and KDEL-TGH

We further investigated whether there were differences in localization of *wt*-TGH versus KDEL-TGH in transfected cells by confocal immunofluorescence microscopy. To investigate the localization of KDEL-TGH versus *wt*-TGH concurrently, we constructed a plasmid containing both myc epitope-tagged KDEL-TGH and flag epitope-tagged *wt*-TGH. Our three-dimensional model of TGH structure (Alam *et al.*, 2002b) as well as the crystal structure of similar carboxylesterases (Bencharit *et al.*, 2002, 2003) show that the C-terminal tail of TGH is relatively unstructured and therefore an ideal target for inclusion of an epitope tag. The epitope tags were placed immediately N-terminal to either –KDEL or –HIEL. Investigation of these tagged proteins expressed individually verified that inclusion of the tags in this location does

not reduce enzyme activity (unpublished data), indicating that tagged TGH is able to fold properly. As expected, the proteins were retained in the ER and were not detected in the culture media. This observation was consistent with previous studies that demonstrated that appending –KDEL or –HIEL to the extreme C-terminus would be both necessary and sufficient to retain these proteins within the ER (Munro and Pelham, 1987; Robbi and Beaufay, 1991). Both epitope-tagged TGH constructs were inserted into the pBudCE4.1 plasmid. This vector has two promoters and two multiple cloning sites, facilitating stable transfection of McArdle RH7777 cells without biases related to the transfection of the two constructs. The KDEL-TGH and *wt*-TGH were immunolocalized by confocal microscopy using antibodies directed against the epitope tags. As shown in Figure 6A, substantial staining of *wt*-TGH is present in a peripheral region in the cell that is devoid of KDEL-TGH. To verify that KDEL-TGH and *wt*-TGH reside in different regions of the ER, we performed confocal immunofluorescence experiments in cells that were expressing KDEL-TGH or *wt*-TGH individually and without epitope tags. Colocalizations were performed with the KDEL-bearing and ER resident protein BiP (also known as Grp78, Munro and Pelham, 1987). Colocalizations with KDEL-TGH and BiP show nearly complete overlap, whereas the overlap is far less extensive between *wt*-TGH and BiP (Figure 6B), supporting the concept that *wt*-TGH is not found in the same regions of the ER as other KDEL-bearing proteins. The staining for *wt*-TGH also appears to be more peripheral than for BiP, which is where the majority of lipid storage droplets are found. Another difference between KDEL and *wt*-TGH that can be discerned from these images is that KDEL-TGH is found in a diffuse pattern, whereas *wt*-TGH is in a lattice pattern.

wt-TGH but not KDEL-TGH Is Found in Regions Containing Neutral Lipid Droplets

After observing differences in the localization of KDEL-TGH versus *wt*-TGH with respect to lipid droplets, we stained transfected McArdle RH7777 cells that had been incubated with or without oleate for 4 h with Nile Red to observe neutral lipid droplets and KDEL-TGH or *wt*-TGH via their epitope tags. The images in Figure 7, A and B, show that the majority of lipid droplets are found in the periphery of the cell where *wt*-TGH is more highly concentrated than KDEL-TGH. Incubation with oleate increased the number of neutral lipid droplets, but did not induce gross changes in TGH protein distribution (unpublished data). Therefore, increased ability of *wt*-TGH to mobilize lipids for secretion on VLDL may be explained by the differences observed in localization and association with lipid droplets. Association with lipid droplets may also explain why TGH cofractionated with ER elements and lipid droplets in previous studies (Lehner *et al.*, 1999).

Cells that were transfected with empty pBudCE4.1 plasmid were used in control experiments that demonstrate staining for both epitope tags was specific. Immunofluorescence was not observed in cells transfected with empty plasmid using either the anti-myc or anti-flag antibodies (unpublished data). Staining for protein disulfide isomerase was used as a reference and a positive control in images of cells transfected with empty plasmid. Transfection of *wt*-TGH or any of the mutants described did not recognizably change the morphology of cells. McArdle RH7777 cells, however, display diversity in morphology among cells on a single dish and in response to culture conditions.

Figure 5. Functional expression of *wt*- and KDEL-TGH in McArdle RH7777 cells. (A) Immunoblots of cell lysates from McArdle RH7777 cells transfected with *wt*-TGH, KDEL-TGH (two independent cell lines), or untransfected cells (McA) after pulse-labeling intracellular lipids with [^3H]oleate and then collecting cells and media after a chase period to investigate secretion of the label. Immunoblots for TGH show relative levels of this protein; immunoblots for TATA-binding protein (TBP) were performed as a gel loading control. (B) Lipase activity in McArdle RH7777 cell lysates using 4-methylumbelliferyl heptanoate (4-MUH) as substrate. Data are the mean $\pm$ SD of six samples and are representative of three independent experiments. (C) Specific activity of *wt*- and KDEL-TGH. The quantity of *wt*-TGH or KDEL-TGH in cell lysates from stably transfected McArdle RH7777 cells was normalized via immunoblots using fluorescent secondary antibodies. Lysates containing equal amounts of TGH were then used in an *in vitro* lipase activity assay with 4-methylumbelliferyl heptanoate (4-MUH) as substrate. Data are the mean $\pm$ SD of three samples. (D–F) Intracellular lipids were labeled with [^3H]oleate and secretion of labeled lipids were probed after a 4-h incubation. Lipids from both media and cells were extracted and separated by TLC. Radioactivity associated with ^3H -labeled triacylglycerol (TG;

panel D), cholesteryl ester (CE; panel E), and phospholipids (PL; panel F) were measured by scintillation counting. Data represent percent of radiolabeled lipid in media/total (cells plus media) and are normalized to cellular protein levels. Data are mean $\pm$ SD of quadruplicate samples and are representative of three independent experiments. For reference, the sum of radiolabel measured in cells and media was an average of 220,234 DPM/mg cell protein for TG, 18,040 DPM/mg protein for CE, and 260,220 DPM/mg for PL in McArdle cells. (D) * $p \leq 0.016$, ** $p \leq 0.018$; (E) *** $p \leq 0.010$, **** $p \leq 0.022$, with respect to cells transfected with *wt*-TGH. (G) Immunogold labeling and electron microscopy of KDEL-TGH in transfected McArdle RH7777 cells. Immunogold labeling of TGH was performed as described in *Materials and Methods*. Magnification, $\times 45,000$. Arrows point to ER elements containing immunogold labeled TGH. M, mitochondria, N, nucleus.

Figure 6. Confocal immunofluorescence microscopy of wt-TGH and KDEL-TGH. (A) McArdle RH7777 cells stably expressing flag-tagged wt-TGH and myc-tagged KDEL-TGH were fixed onto microscope coverslips and incubated with a monoclonal mouse anti-flag antibody and polyclonal rabbit anti-myc antibodies as described in *Materials and Methods*. The flag-tagged wt-TGH and myc-tagged KDEL-TGH were visualized with secondary anti-mouse and anti-rabbit antibodies conjugated to Texas Red and fluorescein or Alexa488, respectively. Images were obtained using a confocal microscope. The boxed region is shown under higher magnification. Bars, 2 μm for higher magnification (left); 5 μm for lower magnification (right). (B) McArdle RH7777 cells stably expressing wt-TGH or KDEL-TGH without epitope tags were fixed onto microscope cover slips as in A. Incubations were performed with a monoclonal mouse anti-BiP antibody and polyclonal rabbit anti-TGH antibodies, which were stained with secondary anti-mouse and anti-rabbit antibodies conjugated to fluorescein and Texas Red, respectively. Images were obtained on a confocal microscope as they were in A.

ApoB-associated Lipids Are Not Substrates for TGH

We have hypothesized that TGH mobilizes TGs in droplets associated with the ER (Gilham *et al.*, 2003). Because VLDL assembly also occurs in the ER, the TGH substrate pool could potentially also include lipids already loaded onto an apoB-containing particle, or assembled VLDL before secretion. To determine if apoB-containing particles are a substrate for TGH, we isolated [³H]oleate-labeled lipoproteins secreted from McArdle RH7777 cells, then transferred them to cells that secrete ΔR-TGH in order to assess the activity of TGH toward apoB-associated lipids. If TGH hydrolyzed VLDL lipids, a time-dependent decrease of radiolabeled lipids from the media would be observed because of uptake of the lipolytic products (fatty acids). As a control, we used microbial lipoprotein lipase (LPL) added exogenously to untransfected McArdle RH7777 cells. LPL is known to hydrolyze TGs on VLDL and other apoB-containing particles such as chylomicrons and LDL (Sugiura and Isobe, 1974). As shown in Figure 8A, there is substantial lipase activity in the

media of ΔR-TGH-transfected cells after 14 h, exceeding the activity seen with 2.5 U of LPL. The amount of labeled TG and CE found in the media, however, were not different between untransfected cells and ΔR-TGH-expressing cells after a 14-h incubation with labeled lipoproteins (Figure 8, B and C). In contrast, incubations that included LPL had markedly reduced amounts of labeled TG and CE in the media. The results indicate that ΔR-TGH does not hydrolyze TG or CE associated with secreted VLDL. The presence of extracellular lipases did not disrupt cell integrity as indicated by the absence of LDH activity in the media (Figure 8D).

Intracellular apoB-containing entities with various folding and lipidation states will develop during assembly of a secretion competent particle (Olofsson *et al.*, 1999; Fisher and Ginsberg, 2002). Delipidation of misfolded or inadequately lipidated particles may be required for retrotranslocation of apoB for degradation by the cytosolic proteasome system (Yao *et al.*, 1997; Olofsson *et al.*, 1999). TGH could recognize these secretion incompetent states and facilitate lipid re-

Figure 7. Colocalization of *wt*-TGH or KDEL-TGH with lipid droplets. (A and B) Images were prepared as they were in Figure 6, except cells were incubated 4 h with oleate before fixation and staining. Nile Red was used to stain neutral lipid droplets, and a monoclonal anti-flag antibody was used to detect flag epitope-tagged *wt*-TGH (A) or polyclonal rabbit anti-myc antibodies utilized to detect myc epitope-tagged KDEL-TGH (B). Images on the right are at higher magnification. Bars, 10 μ m for lower magnification (left); 2 μ m for lower magnification (right).

moval. To assess the ability of TGH to hydrolyze radiolabeled lipids from intracellular apoB, we incubated primary rat hepatocytes with [3 H]oleate, isolated microsomes, immunoprecipitated apoB-containing particles, and then incubated these with TGH and LPL. The amount of lipase activity provided by TGH or LPL was normalized in assays using MUH as substrate. As shown in Figure 9, TGH was not able to hydrolyze lipids associated with intracellular apoB, whereas LPL dramatically reduced levels of TG and liberated free fatty acids (FA).

DISCUSSION

TGH was shown to be glycosylated with a single N-glycosylation site on asparagine 79 (Alam *et al.*, 2002a). N-linked glycosylation is required for proper folding of many proteins because it can facilitate the interaction with chaperones that bind to the carbohydrate moiety, assisting folding and isomerization especially with respect to disulfide bond formation (reviewed in Ellgaard *et al.*, 1999). Glycosylation has also been shown to be required for maximum *in vitro* catalytic activity for other carboxylesterases (Kroetz *et al.*, 1993). A rabbit carboxylesterase very similar to TGH has been

crystallized recently in a fully glycosylated form and in the presence of a known substrate molecule (Bencharit *et al.*, 2002). This enzyme shares the N-glycosylation site at asparagine 79 with TGH, and has an additional N-glycosylation site at asparagine 389. The crystal structure indicates that two *N*-acetyl glucosamine groups are found on asparagine 79; this pattern is likely conserved in TGH. Recently the crystal structure of the first human carboxylesterase was published (Bencharit *et al.*, 2003). The enzyme is known as egasyn, and the resulting structural data indicate the glycosylation pattern at asparagine 79 also consists of two *N*-acetyl glucosamines. Treatment of hepatoma cells expressing a secreted but active mutant of TGH with tunicamycin did not alter the activity of the secreted protein or the secretion of albumin, a nonglycosylated secreted protein. Mutation of the glycosylation site confirmed that TGH does not require this modification for catalytic activity. Lipase activity assays with normalized amounts of *wt*-TGH and the glycosylation mutant indicate lack of glycosylation does not affect specific activity, and the mutant is likely folded properly in a nonglycosylated state (Figure 3C). Therefore, glycosylation appears to be a dispensable modification to TGH.

Figure 8. TGH does not utilize lipids from secretion-competent apoB-containing lipoproteins. (A) Lipase activity was assayed in media of McArdle RH7777 cells stably transfected with Δ R-TGH or untransfected McArdle RH7777 cells $\pm$ microbial lipoprotein lipase (LPL; number or enzymatic units, U, are indicated) after a 14-h incubation. 4-Methylumbelliferyl heptanoate (4-MUH) was used as a substrate. Data are the mean $\pm$ SD of six samples and are representative of three independent experiments. (B and C) Media containing radiolabeled lipoproteins were collected from McArdle RH7777 cells as described in *Materials and Methods*. The amount of radiolabeled triacylglycerol (TG; panel B) and cholesteryl ester (CE; panel C) remaining in media containing labeled lipoproteins was assessed after a 14-h incubation on untransfected McArdle RH7777 cells (control), untransfected McArdle RH7777 cells supplemented with 1.2 U LPL, or on McArdle RH7777 cells stably transfected with Δ R-TGH. For reference, the radiolabel associated with TG in the media of control cells after the 14-h incubation was an average of 7415 DPM and 2570 DPM for CE. (B) * $p \leq 0.0006$; (C) ** $p \leq 0.044$, with respect to control. (D) Lactate dehydrogenase (LDH) activity was assayed in both cells and media of McArdle RH7777 cells (untransfected or stably expressing Δ R-TGH) after 14-h incubation in DMEM $\pm$ 1.2 or 2.5 U of microbial lipoprotein lipase (LPL). LDH activity is represented as the percent of activity in media relative to total (cells and media). To induce cell damage and LDH leakage, some cells were incubated for 2 h with 400 μ M *tert*-butyl hydroperoxide (*t*-BHP).

Soluble proteins that are resident in the lumen of the ER are retrieved from the traffic of secreted proteins from an early Golgi compartment (reviewed in Pelham, 1991). In animal cells, this process is mediated by a C-terminal consensus sequence KDEL (Munro and Pelham, 1987). Several liver microsomal carboxylesterases from human, rat, and rabbit carry deviants of the KDEL consensus ER retention sequence at their extreme C-termini of the type HXEL that have been shown to confer ER retention (Robbi and Beaufay, 1991). Higher eukaryotes appear to tolerate considerable variation at the "X" residue. Why these proteins (and specifically this family of proteins) have diverged from carrying the consensus KDEL sequence for ER retention is presently unclear. The human TGH enzyme bears HIEL at its extreme C-terminus. Expression of a secreted and functional carboxylesterase has been accomplished by mutation of similar C-terminal sequences (Robbi and Beaufay, 1991; Scott *et al.*, 1999; Oosterhoff *et al.*, 2002). To determine if the HIEL motif is the only determinant of TGH ER retention in mammalian cells, we produced a deletion mutant via PCR that lacked the coding region for these amino acids (Δ R-TGH). Our results

showed that this region does confer ER retention without compromising catalytic function.

The subcellular localization of TGH may not only be vital for folding, but also to direct the fate of fatty acids released by TGH-catalyzed lipolysis. Hormone-sensitive lipase (HSL) is a cytosolic enzyme involved in the hydrolysis of stored TG in adipose tissue (Stralfors *et al.*, 1987). When HSL is ectopically expressed in hepatoma cells, the liberated fatty acids are directed toward oxidation (Pease *et al.*, 1999) rather than secretion as seen with TGH (Lehner and Vance, 1999).

The C-terminal sequence deleted from TGH in order to produce a secreted enzyme may be functionally relevant in directing TGH to a subdomain of the ER. To explore this possibility, we created a mutant of TGH that carries the Δ -KDEL C-terminal sequence rather than the endogenous Δ -HIEL sequence. We then assessed the ability of expressed TGH to mobilize intracellular lipids in transfected cells and to colocalize with other soluble proteins in the ER lumen and with neutral lipid droplets. Because *wt*-TGH-expressing cells secreted more neutral lipid than KDEL-TGH-expressing cells, we speculate that this C-terminal sequence not only

Figure 9. TGH does not hydrolyze lipids from intracellular apoB-containing particles. Lipids in primary rat hepatocytes were labeled by incubation with [3 H]oleate. Microsomes were prepared and carbonate extracted to release luminal contents and peripheral membrane proteins. Liberated apoB-containing particles were isolated by immunoprecipitation of apoB and then incubated without any treatment, with media alone (DMEM), or with ΔR-TGH or microbial lipoprotein lipase (LPL) containing an equal amount of lipase activity for 14 h. Lipids were extracted, separated by TLC and radioactivity associated with each lipid species determined by scintillation counting. Shown are radioactivities associated with cholesteryl ester (CE), triacylglycerol (TG), free fatty acids (FA), and phospholipids (PL). For reference, the radiolabel associated with TG in control incubations after 14 h was an average of 2126 DPM. Data are the mean $\pm$ SD of three samples.

confers retention in the ER, but also concentrates this protein in a microdomain of this organelle where TGH may more readily access its substrate or where the majority of VLDL assembly occurs, allowing more efficient secretion of lipids on apoB. This hypothesis is supported by our observation of nonuniform cellular distribution of *wt*- and KDEL-TGH in the ER elements. Confocal immunofluorescence analyses showed *wt*-TGH in unidentified projections near the cell surface that contained little or no KDEL-TGH. The increased ability of *wt*-TGH to mobilize lipid droplets for VLDL assembly compared with that of KDEL-TGH may be attributed to better access to this substrate because the majority of lipid droplets are localized near the periphery of the cells.

It is established that the ER is a diverse environment with specialized activities sequestered in particular regions. Examples include regions for ribosome attachment (i.e., smooth vs. rough ER), regions for vesicle docking and budding, and regions surrounding the nucleus that contain nuclear pores. It is presently unclear how proteins involved in different activities are delivered to these specific regions of the ER. Similarly, it is feasible that a region of the ER exists that is specialized in lipoprotein assembly. The mechanism of how TGH is delivered to this region remains uncertain, but appears to involve the C-terminal –HIEL sequence. To our knowledge, this is the first time a putative sequence culminating in specific trafficking of proteins within the ER has been identified. Potentially a receptor other than the well-characterized KDEL receptor (Lewis and Pelham, 1990; Tang *et al.*, 1993; Wilson *et al.*, 1993) is involved in retrieval of soluble proteins bearing C-terminal sequences like –HIEL from the Golgi apparatus and delivers them to this location of the ER. Alternately, TGH may associate with proteins within the ER that sequester it to regions in proximity to lipid droplets or regions involved in lipoprotein assembly. The latter mechanism would require an interaction dependent on the C-terminal sequence, or it would also function for the KDEL-TGH mutant. It has been proposed that the exit of the assembled VLDL particle from the ER may also require specialized machinery as VLDLs are much larger than transport vesicles (Schekman and Mellman, 1997). This machinery may also be localized to this region of VLDL assembly by the same mechanism as TGH.

Recent reports have demonstrated the involvement of Sar1b in the trafficking of apoB-containing particles between the ER and the Golgi apparatus (reviewed in Brodsky *et al.*, 2004; Shoulders *et al.*, 2004). Sar1b is an ER-derived GTPase involved in COPII-mediated vesicle formation, and muta-

tions in this protein have been shown to be responsible for an apoB-related trafficking disorder known as Anderson's disease (Jones *et al.*, 2003). It remains to be determined if Sar1b is required for budding of apoB-containing particles from the ER or for docking with the Golgi (Gusarova *et al.*, 2003; Siddiqi *et al.*, 2003; Brodsky *et al.*, 2004). ApoB is cotranslationally lipidated in the ER to form a primordial particle that matures into a VLDL particle (Olofsson *et al.*, 1999; Shelness and Sellers, 2001). Here we show that apoB-associated lipids are not a substrate pool for TGH. This was unexpected because apoB and TGH can be cross-linked in microsomes isolated from primary rat hepatocytes, indicating a possible interaction between these two proteins (Rashid *et al.*, 2002; Gilham and Lehner, unpublished results). In addition, we have observed partial colocalization between apoB and TGH in confocal images taken from TGH-transfected McArdle RH7777 cells, suggesting their coexistence in the same compartment during initial apoB-containing lipoprotein assembly (Gao and Lehner, unpublished results). Previous studies have shown that TGH activity enhances VLDL lipidation in transfected hepatoma cell lines (Lehner and Vance, 1999), signifying that apoB-associated TG is not a substrate for TGH within the ER. Furthermore, inhibition of TGH activity did not lead to the accumulation of intracellular apoB, suggesting that the role of TGH is not delipidation of misfolded apoB (Gilham *et al.*, 2003).

TG and other neutral lipids forming the core of the intracellular storage droplets are surrounded by a monolayer of phospholipids and coat proteins (reviewed in Londos *et al.*, 1999 and Murphy, 2001). These coat proteins may serve a structural role to maintain the lipid droplet integrity, prevent fusion with any adjacent lipophilic surface or have a functional role as docking sites for lipogenic or lipolytic enzymes. We have hypothesized that the TGH substrate pool in hepatocytes exists in an ER luminal lipid droplet whose production involves the action of microsomal triglyceride transfer protein (Gilham *et al.*, 2003). Lipid droplet coat protein(s) in the ER lumen of hepatocytes, such as apoB or other apolipoproteins, may modulate TGH access and TG mobilization. ApoB may not be a compatible coat, so TGH cannot hydrolyze the associated lipids. Such a system would allow regulation of both the lipase activity and its ability to access substrate via modifications to the lipase and/or the coat proteins.

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