Mutation of F_{417} but not of L_{418} or L_{420} in the lipid binding domain decreases the activity of triacylglycerol hydrolase

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Abstract **Human triacylglycerol hydrolase (hTGH) has been shown to play a role in hepatic lipid metabolism. Triacylglycerol hydrolase (TGH) hydrolyzes insoluble carboxylic esters at lipid/water interfaces, although the mechanism by which the enzyme adsorbs to lipid droplets is unclear. Three-dimensional modeling of hTGH predicts that catalytic residues are adjacent to an α-helix that may mediate TGH/lipid interaction. The helix contains a putative neutral lipid binding domain consisting of the octapeptide FLDLIADV (amino acid residues 417–424) with the consensus sequence FLXLXXXn (where n is a nonpolar residue and X is any amino acid except proline) identified in several other proteins that bind or metabolize neutral lipids. Deletion of this α-helix abolished the lipolytic activity of hTGH. Replacement of F_{417} with alanine reduced activity by 40% toward both insoluble and soluble esters, whereas replacement of L_{418} and L_{420} with alanine did not. Another potential mechanism of increasing TGH affinity for lipid is via reversible acylation. Molecular modeling predicts that C_{390} is available for covalent acylation. However, neither chemical modification of C_{390} nor mutation to alanine affected activity. Our findings indicate that F_{417} but not L_{418}, L_{420}, or C_{390} participates in substrate hydrolysis by hTGH.**—Alam, M., D. Gilham, D. E. Vance, and R. Lehner. **Mutation of F_{417} but not of L_{418} or L_{420} in the lipid binding domain decreases the activity of triacylglycerol hydrolase.** ***J. Lipid Res.*** **2006.** 47: **375–383.**

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Several endoplasmic reticulum-associated carboxylesterases with broad and overlapping substrate specificities have been identified (1–5). Mammalian carboxylesterases are known to metabolize numerous analgesic and narcotic compounds, including aspirin, cocaine, heroin, procaine, and meperidine (6–9). Hepatic carboxylesterases are involved in the biotransformation of xenobiotics and natural substrates by hydrolyzing compounds containing ester, thioester, or amide bonds (10). Carboxylesterases are also used to activate anticancer prodrugs such as CPT-11 (irinotecan) (11, 12).

Our laboratory has investigated the participation of hepatic carboxylesterases in lipid metabolism. We have purified a carboxylesterase that catalyzes the lipolysis of triacylglycerols and termed it triacylglycerol hydrolase (TGH) (13–15) and have isolated and expressed a cDNA for human triacylglycerol hydrolase (hTGH GenBank accession number NM_001266) (16). Hepatic TGH participates in the mobilization of stored triacylglycerol (TG) for the assembly of VLDL (14,17–20). Because VLDL is the precursor for plasma LDL and a high level of plasma LDL is a major risk factor for the development of atherosclerosis and coronary heart disease (21, 22), investigations of the mechanism of action of enzymes and proteins involved in the assembly and secretion of VLDL are of clinical importance. Hepatic VLDL assembly is predominantly regulated by the provision of lipid (23–25). It has been reported by several groups that the majority of TG in VLDL originates from cytoplasmic storage pools, and mobilization of this TG involves lipolysis followed by reesterification to reform TG before loading onto nascent apolipoprotein B-containing particles (26–30). Therefore, TGH is a pharmacological target (inhibition) to decrease plasma lipid levels, and detailed knowledge of the structure-activity relationships will assist in generating specific hTGH inhibitors.

Lipid-binding sequences have been proposed in proteins that interact with or metabolize lipids. TGH readily translocates from the aqueous milieu to diacylglycerol monolayers (13) and has been found associated with cytoplasmic lipid droplets (17) however, the regions on the protein that confer TGH affinity for lipids have not been characterized. Au-Young and Fielding (31) proposed a neutral lipid binding domain (NLBD) in human plasma cholesteryl ester transfer protein (hCETP). This putative lipid binding octapeptide forms an amphipathic α-helix with the consensus sequence FLXLXXXn (where n is a

Abbreviations: hCETP, human plasma cholesteryl ester transfer protein; hTGH, human triacylglycerol hydrolase; 4MUH, 4-methylumbelliferyl heptanoate; Ni-NTA, nickel-nitrilotriacetic acid; NLBD, neutral lipid binding domain; PNP, *p*-nitrophenyl; TG, triacylglycerol; TGH, triacylglycerol hydrolase.

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nonpolar residue and X is any amino acid except proline). By sequence alignment, we identified a putative NLBD motif in porcine (13) and human (32) TGH.

It was reported recently that a porcine carboxylesterase homologous to hTGH may undergo cysteine acylation (33). Because the acylation of cysteine residues is reversible, an attractive hypothesis has emerged that adsorption of hTGH to lipids could be mediated by this posttranslational modification. The deduced amino acid sequence of hTGH has five cysteine residues. Our three-dimensional model structure suggests that two disulfide bridges are formed between C_{87}–C_{116} and C_{274}–C_{285} (32). The remaining cysteine residue at position 390 would be available for modification and appears to be surface-oriented.

In this study, we explored the possible roles of the putative NLBD and C_{390} residues in hTGH catalysis using site-directed mutagenesis and biochemical approaches.

TABLE 1. Oligonucleotides for site-directed mutagenesis of hTGH

Oligonucleotide	Sequence (5′–3′)
Δ410–424 forward	gga gga aca gac gac atg ttt ggt gtc cca tct gtg att gtg g
Δ410–424 reverse	cca caa tca cag atg gga cac caa aca tgt cgt ctg ttc ctc c
F_{417}A forward	gtc aaa aag aaa gac ctg **gcc** ctg gac ttg ata gca gat g
F_{417}A reverse	cat ctg cta tca agt cca g**ggg** **c**ca ggt ctt tct ttt tga c
L_{418}A forward	gtc aaa aag aaa gac ctg ttc **gcg** gac ttg ata gca gat g
L_{418}A reverse	cat ctg cta tca agt c**cg** **c**ga aca ggt ctt tct ttt tga c
L_{420}A forward	gaa aga cct gtt cct gga c**gc** **g**at agc aga tgt gat gtt tgg tg
L_{420}A reverse	cac caa aca tca cat ctg cta t**cg** **c**gt cca gga aca ggt ctt tc
C_{390}A forward	cct atc ccc ttg tt**g** **cc**a ttg cta agg aac tga ttc c
C_{390}A reverse	gga atc agt tcc tta gca at**g** **gc**a aca agg gga tag g

hTGH, human triacylglycerol hydrolase. Mismatches with the template are indicated by boldface letters.

MATERIALS AND METHODS

Materials

Iodoacetamide, *p*-nitrophenyl (PNP) acetate, PNP butyrate, PNP laurate, *p*-nitrophenol, 4-methylumbelliferyl heptanoate (4MUH), 4-methylumbelliferone, anti-flag M2 antibody attached to agarose beads, and BSA were from Sigma-Aldrich (Oakville, Ontario, Canada). SF-900 II SFM medium, FBS, DMEM, Lipofectamine 2000, penicillin, streptomycin, and geneticin (G-418 sulfate) were from Invitrogen (Burlington, Ontario, Canada). Restriction endonucleases and T4-DNA ligase for subcloning of cDNAs were from New England Biolabs (Mississauga, Ontario, Canada) or Boehringer Mannheim (Laval, Québec, Canada). Nickel-nitrilotriacetic acid (Ni-NTA) resin for purification of recombinant polyhistidine-tagged proteins and the anti-His4 monoclonal antibody were from Qiagen (Valencia, CA). Nitrocellulose membranes, SDS-PAGE reagents, and prestained protein molecular mass markers were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Unless specified otherwise, all other reagents were from local suppliers and of the highest quality available.

Mutagenic oligonucleotides and site-directed mutagenesis

All PCRs for site-directed mutagenesis were performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Scarborough, Ontario, Canada). Oligonucleotides were synthesized by the Institute for Biomolecular Design at the University of Alberta. The oligonucleotides used to generate mutant hTGH constructs are listed in **Table 1**. Site-directed mutagenesis was performed using the Quikchange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) in accordance with the manufacturer's protocol.

Recombinant plasmids

We initiated this study with a full-length hTGH cDNA construct with a C-terminal polyhistidine tag in the pFASTBAC plasmid (Invitrogen) described previously (16). The polyhistidine-tagged protein remains intracellular in Sf9 cells, despite the prediction that it would be secreted (16). This plasmid was the template for mutagenesis to generate the Δ410–424 and C_{390}A mutants using the primer pairs listed in Table 1. Recombinant baculovirus was generated from these plasmids as described for the BAC-TO-BAC baculovirus system (Invitrogen) and used for the expression of mutant and wild-type (wt)-hTGH in Sf9 cells as described below.

The point mutations F_{417}A, L_{418}A, and L_{420}A were also generated by PCR-based mutagenesis using the primers indicated in Table 1. The template used was a deletion mutant of hTGH lacking the coding region for the C-terminal sequence HIEL (20) with an additional polyhistidine tag appended to the C terminus to facilitate purification. The construct hTGHΔHIEL-His6 was cloned in the pCI-neo mammalian expression vector (Promega Corp., Madison, WI). The new plasmids generated by mutagenesis were used for expression in Cos-7 cells as described below.

Expression of recombinant hTGH and its mutants in Sf9 cells

Sf9 cells were cultured at 28°C with GIBCO BRL Sf-900 II supplemented with 5% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Expression of hTGH in Sf9 cells using recombinant baculovirus was performed as described previously (32). Briefly, for expression of wt-hTGH, Δ410–424, and hTGH with the point mutation C_{390}A, 1×10^6 Sf9 cells were seeded in 100 mm tissue culture dishes. Cells were infected with recombinant baculovirus at a multiplicity of infection of 1–5, incubated for 72–96 h after infection, harvested in PBS, and analyzed for protein content with Bio-Rad protein assay reagent using BSA as a standard. Membrane and cytosolic fractions were prepared from a postmitochondrial supernatant as described previously (32).

Expression of hTGH was assessed by immunoblotting the cell lysates using polyclonal anti-hTGH antibodies produced in our laboratory (16). The nitrocellulose membranes were stained with Ponceau S.

Point mutations in the hTGH NLBD and expression in Cos-7 cells

Cos-7 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in 5% CO_2 in DMEM containing 10% FBS. For transfections, cells were seeded on 60 mm plates and grown to 80–90% confluence, then 4 μg of plasmid DNA containing hTGHΔHIEL-His6 point mutant constructs (F_{417}A, L_{418}A, or L_{420}A) and hTGHΔHIEL-His6 were introduced using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, the media were replaced with fresh DMEM without FBS, and incubation continued for an additional 48 h. Media were collected and cells were washed twice with ice-

cold PBS, harvested in 2 ml of PBS, and sonicated. Protein content was measured as described above.

Affinity isolation of recombinant polyhistidine-tagged hTGH proteins from Cos-7 culture media

Transient transfection of Cos-7 cells resulted in the secretion of polyhistidine-tagged recombinant hTGH into the cell culture media. Media were adjusted to contain a final concentration of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 5 mM imidazole (binding buffer). Ni-NTA agarose resin was added (125 μl/ml media), and the suspension was incubated at 4°C for 2 h on a rotating rack to capture the hTGH protein present. Samples were centrifuged at 14,000 rpm in a bench-top microcentrifuge for 5 min, and the supernatants were removed. The Ni-NTA resin was washed with binding buffer. For the analysis of hTGH bound to the Ni-NTA, the resin was treated with denaturing SDS-PAGE sample buffer to liberate bound proteins. The proteins were resolved in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The presence of hTGH was analyzed using polyclonal anti-hTGH antibodies (16, 32) and detected by chemiluminescence. The membranes were stripped of antibodies at 50°C in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol, washed with TTBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20), blocked with 5% milk in TTBS, and reprobed with monoclonal anti-His4 antibody (16, 32).

In vitro fatty acylation of hTGH

Microsomes were prepared as described previously (13) from McArdle RH7777 cells stably expressing hTGH with a flag epitope tag engineered just before the C-terminal endoplasmic reticulum retrieval sequence HIEL (20). Therefore, the expressed flag-tagged hTGH is retained in this organelle. Investigation of microsomal protein acylation was performed essentially as described (34) with some modifications. In brief, 125 μg of microsomal protein was incubated with 0.5 μCi of [^{14}C]oleoyl-CoA (Amersham Life Sciences, Baie d'Urfé, Québec, Canada) in 50 mM Tris-HCl (pH 7.6) in a final volume of 100 μl at 37°C for 30 min. Membranes were solubilized with 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA (immunoprecipitation buffer) containing Complete Protease Inhibitor Cocktail (Roche), and the solubilized proteins were resolved from insoluble debris by centrifugation. Although palmitate is the fatty acid most commonly found on cysteine-acylated proteins, cysteine acylation is not specific for palmitate and other fatty acyl moieties can be found, including cysteine oleoylation (35). hTGH was immunoprecipitated from supernatants with 30 μl of anti-flag M2 antibody attached to agarose beads overnight at 4°C on a rotating rack. The immunoprecipitates were washed three times with immunoprecipitation buffer. Proteins remaining in the supernatants after immunoprecipitation were precipitated with trichloroacetic acid (36). Proteins in trichloroacetic acid and immunoprecipitates were denatured in nonreducing sample buffer (without β-mercaptoethanol), separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Gels also contained ^{14}C-labeled methylated protein standards (Amersham Life Sciences). The membranes were probed for the presence of hTGH with anti-hTGH polyclonal antibodies. After immunoblotting, the membranes were exposed to a Bio-Rad phosphorimaging screen, and the radioactivity covalently associated with proteins was analyzed using a Bio-Rad Molecular Imager FX Phosphorimager. Finally, proteins on the membrane were stained with Ponceau S, the areas corresponding to hTGH and labeled control proteins were excised, and the associated radioactivity was determined by scintillation counting.

Measurements of lipase and esterase activities

Colorimetric assays of esterase and lipase activities with PNP acetate, butyrate, and laurate were carried out essentially as described previously (13, 16). Briefly, protein samples were incubated in a 0.2 ml assay solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.01% Triton X-100, and 250 μM PNP ester substrate. After an incubation period, the release of PNP was measured on 96-well clear microtiter plates as the absorbance at 405 nm on a Molecular Devices SpectraMax 250 spectrophotometer (37). Activity is expressed as nanomoles of PNP released per milligram of protein or milliliter of Cos-7 cell culture media per minute. Alternatively, a fluorescence-based assay was used that employs water-insoluble 4MUH as a substrate, as described previously (20,37). Activity is expressed as nanograms of 4-methylumbelliferone released per milligram of protein or milliliter of Cos-7 cell culture media per minute using 4-methylumbelliferone (sodium salt) as a standard. TGH exhibits interfacial kinetics with both PNP laurate (16) and 4MUH, and the assay conditions were optimized to measure maximal hydrolytic rates.

For chemical modification of the free cysteine residue present in hTGH, purified wt-hTGH was treated for 45 min with iodoacetamide at the indicated concentrations on ice before assessment of the lipolytic activity.

RESULTS

Deletion of putative NLBD results in inactive hTGH

TGH hydrolyzes various carboxylic esters, including water-insoluble lipids (13, 14, 32). Details regarding how TGH engages lipid substrates remain obscure. It has been proposed (31) that a NLBD, found in a variety of proteins that interact with or metabolize neutral lipids, could potentially promote this interaction. The NLBD motif was originally described in human cholesteryl ester transfer protein (31) but is also found in porcine TGH (13), human lecithin:cholesterol acyltransferase (38), human cholesterol esterase (39), rat hormone-sensitive lipase (40), and human cholesterol 7α-hydroxylase (41). A consensus NLBD sequence (F_{417}LDLIADV$_{424}$) has been identified in hTGH (**Table 2**) and is contained within an α-helix adjacent to a hydrophobic entry pocket leading to buried catalytic residues (32, 42). Thus, the NLBD may regulate or facilitate interaction with hydrophobic surfaces such as membranes or substrate pools such as intracellular lipid droplets.

TABLE 2. Alignment of putative neutral lipid binding domains

Protein	Sequence	Position	Reference
hTGH	**F L** D **L** I A D V	417–424	32
pTGH	**F L** D **L** M G D V	416–423	13
hCETP	**F L** L **L** Q M D F	454–461	31
hLCAT	**C L** H **L** L Y F L	184–191	38
hCEase	**C L** Y **L** N I W V	80–87	39
rHSL	**F L** S **L** A A L C	734–741	40
hCYP7α	**F L I L** M l S Y	457–464	41
Consensus	**F/C L** X **L** X X X n	N/C terminus	

pTGH, porcine triacylglycerol hydrolase hCEPT, human cholesteryl ester transfer protein hLCAT, human lecithin:cholesterol acyltransferase hCEase, human cholesterol esterase rHSL, rat hormone-sensitive lipase hCYP7a, human cholesterol 7α-hydroxylase. Conserved residues are shown in boldface, a nonpolar residue is indicated with n, and any amino acid except proline is indicated with X.

We previously used baculovirus expression systems to generate wild type and various mutants of hTGH in Sf9 insect cells (16, 32). In this study, we generated and expressed a hTGH deletion mutant that lacked the entire α-helical segment comprising residues 410–424(Δ410–424) encompassing the NLBD. Baculovirus containing this mutant was generated and used to infect Sf9 cells. The Δ410–424 hTGH mutant was expressed to a similar level as wt-hTGH, as indicated by immunoblotting (**Fig. 1A**). The deletion mutant appeared to migrate with a slightly lower molecular mass on the 10% polyacrylamide gel as a result of the deletion of 14 amino acids. All of the expressed Δ410–424 hTGH was retained in the membrane fraction of Sf9 cells (results not shown). Comparisons of lipolytic activities of noninfected Sf9 cell extracts with cell extracts containing Δ410–424 hTGH showed that the mutant was not active, because similar levels of expression of wt-hTGH yielded an ~4-fold increase of lipolytic activity in the Sf9 cell lysates (Fig. 1B). The lack of lipolytic activity of the deletion mutant could be attributable either to a crucial role of the NLBD for catalytic events or to a detrimental effect of deleting these residues that results in improper folding. To assess the latter possibility, we attempted to purify the expressed deletion mutant by detergent solubilization of the protein from Sf9 microsomes and purification by Ni-NTA affinity chromatography for circular dichroism spectral analysis, a procedure we successfully used for the purification of hTGH active site mutants (32). However, we did not succeed in solubilizing the Δ410–424 hTGH mutant from Sf9 cell microsomes, suggesting that the mutant was misfolded. Therefore, we addressed the function of the putative NLBD by point mutagenesis rather than multiple amino acid deletion.

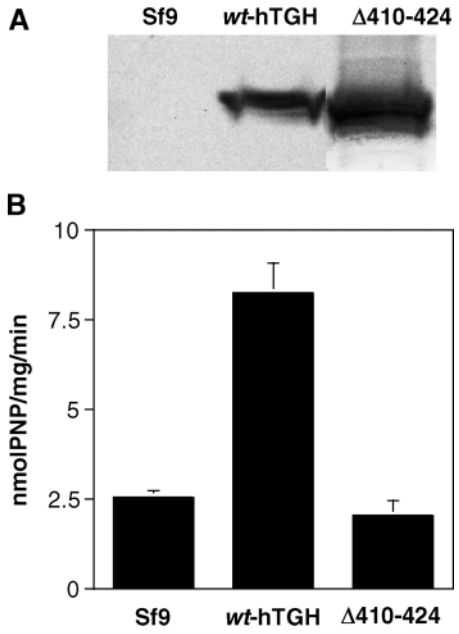

Fig. 1. Expression of the human triacylglycerol hydrolase (hTGH) neutral lipid binding domain (NLBD) deletion mutant (Δ410–424). A: Homogenate (100 μg of protein) from uninfected Sf9 cells, cells infected with wild-type hTGH (wt-hTGH), and cells infected with Δ410–424 were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane and immunoblotting with anti-hTGH polyclonal antibodies. B: *p*-Nitrophenyl (PNP) laurate hydrolysis by 50 μg of cell homogenate protein from uninfected Sf9 cells and cells expressing similar amounts of wt-hTGH and Δ410–424. Data are the mean ± SD of triplicate experiments.

Expression of wt-hTGH and hTGH point mutants in Cos-7 cells

The conserved phenylalanine and leucine residues present within the putative NLBD (Table 2) have been proposed to provide the necessary hydrophobicity for interaction with lipids (31). In hTGH, these NLBD conserved residues are F_{417}, L_{418}, and L_{420} and were the targets for point mutations. To monitor proper folding of the mutants in Cos-7 cells, we used a hTGH construct that lacks the C-terminal HIEL sequence, resulting in a secreted protein (20). Therefore, if a mutant hTGH is secreted, that strongly suggests proper folding because the protein passed the stringent quality control of protein processing in the endoplasmic reticulum (43–46). The constructs were also tagged with six histidine residues at the C terminus to facilitate purification by affinity chromatography. The cDNAs were cloned into the pCI-neo mammalian expression vector, and upon transient expression of the hTGHΔHIEL-His6 point mutants (F_{417}A, L_{418}A, and L_{420}A) or hTGHΔHIEL-His6 in Cos-7 cells, the hTGH proteins were captured from the culture media on Ni-NTA resin via the C-terminal polyhistidine tag. We chose to substitute the residues with alanine because this substitution would be expected to retain the helical structure of the domain but would decrease its hydrophobicity, which we hypothesized would affect the affinity of the hydrolase for lipids. The amounts of the hTGH proteins captured from the media using affinity chromatography were assessed by immunoblotting with anti-hTGH polyclonal antibodies and an anti-His4 monoclonal antibody (**Fig. 2A,** B). Only marginal amounts of the hTGH proteins could be detected in cell lysates by immunoblotting and lipase activity assays, indicating efficient secretion (results not shown). Lipase/esterase activity in the media was determined using substrates with different carbon chain lengths. As shown in Fig. 2C, the L_{418}A and L_{420}A mutants did not show any significant changes in lipolytic activity with the insoluble lipase substrate 4MUH, and the mutants retained the same level of activity as hTGHΔHIEL-His6. However, ~40% of the lipolytic activity was lost in the F_{417}A mutant compared with hTGHΔHIEL-His6, L_{418}A, and L_{420}A. Significant reduction of hydrolytic activity by the F_{417}A mutant was also seen with two soluble esterase substrates, PNP acetate and butyrate, whereas L_{418}A and L_{420}A continued to retain full catalytic activity regardless of substrate carbon chain length (Fig. 2D, E). We conclude that F_{417} is important for the efficient hydrolysis of all substrates, including insoluble esters.

C_{390} in hTGH is not involved in substrate binding/catalysis

The hTGH primary amino acid sequence contains five cysteine residues (C_{87}, C_{116}, C_{274}, C_{285}, and C_{390}). Two di-

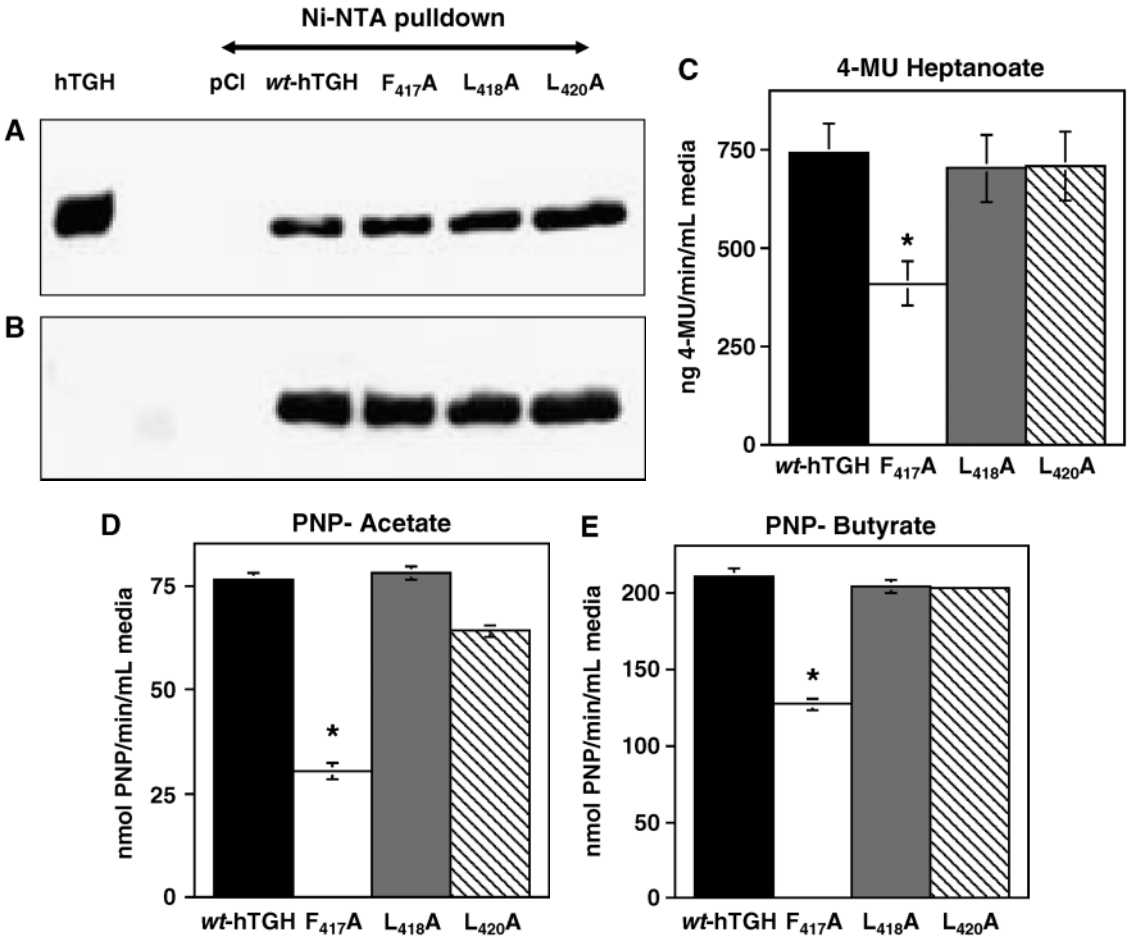

Fig. 2. Expression and analysis of hTGHΔHIEL-His6 (wt-hTGH) and the indicated NLBD point mutants secreted into the culture media from transiently transfected Cos-7 cells. Equal volumes of culture media were mixed with nickel-nitrilotriacetic acid (Ni-NTA) resin to allow the binding of expressed histidine-tagged proteins as described in Materials and Methods. After washing, the Ni-NTA resin was treated with SDS sample buffer, and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes followed by immunoblotting. A: Immunoblot with anti-hTGH polyclonal antibodies. B: Immunoblot with anti-His4 monoclonal antibodies. C–E: Hydrolase activity in culture media of Cos-7 cells containing the same immunodetectable amounts of hTGHΔHIEL-His6 (wt-hTGH) and the indicated mutant constructs using 4-methylumbelliferyl (4-MU) heptanoate (C), PNP acetate (D), and PNP butyrate (E) as substrates. Values were corrected for endogenous hydrolytic activity by subtraction of activities obtained from transfections with empty pCI-neo plasmid (pCI). * $P < 0.01$ compared with wt-hTGH control. Data are the mean ± SD of triplicate experiments.

sulfide bridges are predicted to form between $C_{87–116}$ and $C_{274–285}$, leaving C_{390} free based on the proposed three-dimensional structure of hTGH (32). C_{390} could be available for the formation of intermolecular disulfide bonds. However, treatment of purified hTGH with or without β-mercaptoethanol did not affect its electrophoretic mobility (**Fig. 3A**), indicating that no covalent intermolecular association via a disulfide bond exists between TGH and itself or other proteins that would give rise to either homodimers or heterodimers. Detection of heterooligomers may have been hindered through expression in Sf9 cells that could lack potential hTGH-interacting proteins however, we observed no high migrating molecular mass hTGH-containing protein complexes on nonreducing SDS-polyacrylamide gels when the protein was expressed in hepatoma McArdle RH7777 cells (Fig. 3A). In addition, treatment of hTGH with the cysteine alkylating agent iodoacetamide did not result in the alteration of hTGH activity, suggesting that C_{390} is not involved in catalysis (Fig. 3B).

It is well known that protein acylation (myristoylation and palmitoylation) influences a wide spectrum of structural and functional features of proteins. These modifications can alter localization, association with membranes (reviewed in 47), or enzyme activity (48). Cysteine acylation is a reversible posttranslational modification (49, 50). It has been reported that a porcine carboxylesterase with high homology to hTGH may undergo cysteine acylation (33). Because hTGH also contains a free cysteine residue, we investigated whether the enzyme undergoes this posttranslational modification. As shown in **Fig. 4A**, incubations of microsomes isolated from McArdle RH7777 cells stably expressing hTGH with [^{14}C]oleoyl-CoA resulted in the acylation of proteins present in the supernatant after immunoprecipitation of hTGH however, the immunoprecipitated hTGH was not acylated. This observation

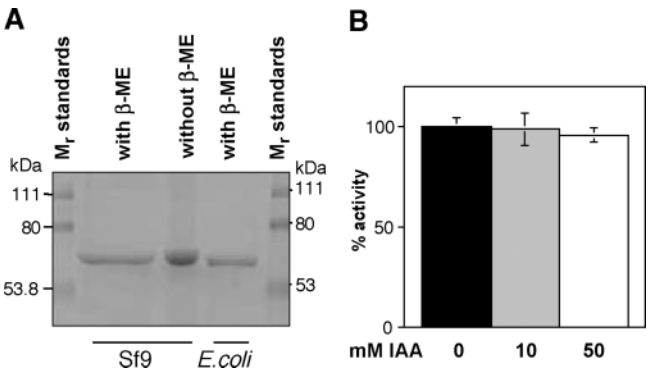

Fig. 3. Effect of β-mercaptoethanol and iodoacetamide (IAA) on the electrophoretic mobility and activity of wt-hTGH. hTGH was purified from baculovirus-infected Sf9 cells under native conditions and subjected to various treatments. A: Purified wt-hTGH expressed in Sf9 cells and in *Escherichia coli* was treated with SDS-PAGE sample buffer with and without β-mercaptoethanol (β-ME) and subjected to SDS-PAGE followed by staining with Coomassie brilliant blue R-250. M_r standards, prestained molecular mass standards (phosphorylase B, 111,000 Da BSA, 80,000 Da ovalbumin, 53,800 Da). B: wt-hTGH purified from Sf9 cells treated with various concentrations of iodoacetamide on ice for 45 min before determination of lipolytic activity. Relative activities using PNP laurate are shown as a percentage of untreated wt-hTGH (100%). Data are the mean ± SD of triplicate experiments.

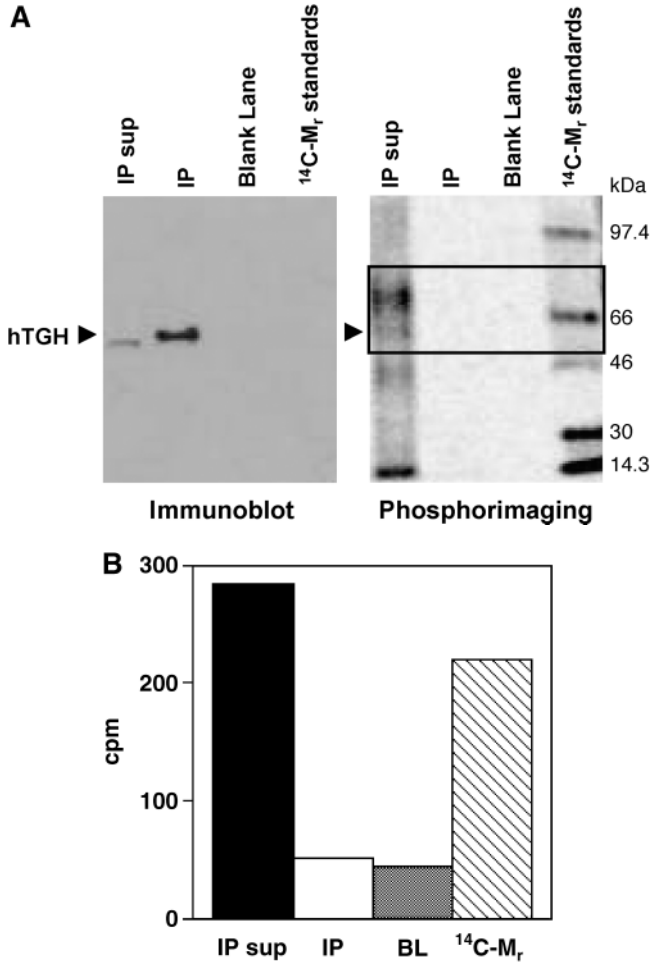

Fig. 4. wt-hTGH is not acylated in McArdle RH7777 cells. Microsomal proteins from McArdle RH7777 cells stably expressing flag epitope-tagged hTGH were incubated with [^{14}C]oleoyl-CoA as described in Materials and Methods followed by immunoprecipitation of hTGH with monoclonal anti-flag M2 antibody. Both immunoprecipitated proteins (IP) and remaining nonimmunoprecipitated microsomal proteins (IP sup) were subjected to nonreducing SDS-PAGE followed by transfer to a nitrocellulose membrane. A: Immunoblot and phosphorimaging. M_r standards, prestained molecular mass standards. B: Radioactivity in the area marked by a box in A associated with immunoprecipitated hTGH (IP), nonimmunoprecipitated proteins (IP sup), blank lane (BL), and ^{14}C-labeled molecular mass standard (^{14}C-M_r) after excision of the areas of the nitrocellulose membrane, followed by scintillation counting.

was confirmed by scintillation counting of the regions of the nitrocellulose membrane containing the hTGH band (Fig. 4B). Therefore, we conclude that C_{390} of hTGH does not play a crucial role in catalysis or in the association with membranes or other substrate pools via acylation. To further support these observations, we constructed a C_{390}A hTGH mutant and expressed the mutant in Sf9 cells using a baculovirus protein expression system and performed lipase activity determinations. **Figure 5A,** B shows that the C_{390}A mutant hTGH protein was expressed in baculovirus-infected Sf9 cells. Activity measurements with PNP laurate in parallel with assays of the same amounts of wt-hTGH indicated no differences in the hydrolysis of the lipid ester (Fig. 5B). These data support our conclusion that C_{390} is not essential for hTGH activity.

DISCUSSION

Hepatic TGH has been shown to participate in the mobilization of cytoplasmic TG for VLDL assembly (14, 18–20). Using site-directed mutagenesis, we have shown that the amino acid residues S_{221}, E_{354}, and H_{468} constitute the catalytic triad (32). Three-dimensional modeling of hTGH based on the crystal structure of acetylcholine esterase (32) indicates the presence of a typical α/β hydrolase fold consistent with known structures of other serine esterases (51). The modeled structure closely resembles the recently published crystal structures of a rabbit liver carboxylesterase (12) and the first published high-resolution structure of a human carboxylesterase (52). Structural data also indicate that subtle differences in the shapes of carboxylesterase active sites influence substrate specificity (42).

Sequence alignments with other neutral lipid-metabolizing and -interacting proteins led to the prediction of a putative NLBD that is involved in interactions with lipid substrates (31). We hypothesized that the NLBD in hTGH is involved in engaging hydrophobic substrates. The NLBD consists of an amphipathic octapeptide with a FLXLXXXn consensus that is conserved among several proteins involved in the transfer or hydrolysis of neutral lipids. It was proposed that in hCETP, the FLLL residues (Table 2) might be required for binding to lipoproteins and effective lipid transfer, possibly in concert with other C-ter-

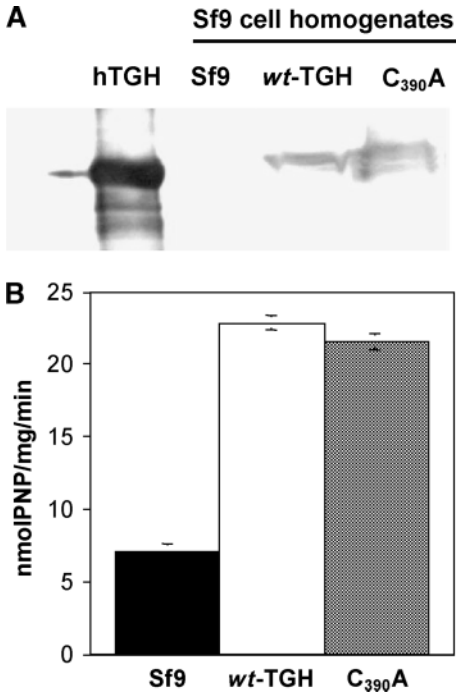

Fig. 5. Expression and activity of hTGH $C_{390}A$ mutant. Homogenates were prepared from uninfected Sf9 cells, cells infected with wt-hTGH, and the hTGH $C_{390}A$ mutant as described in Materials and Methods. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. A: Membrane immunoblotted with anti-hTGH polyclonal antibodies. B: Activity profile of cell homogenates (100 μg of protein) from uninfected and infected Sf9 cells (wt-hTGH or C390A mutant) using PNP laurate as substrate. Data are the mean ± SD of triplicate experiments.

minal sequences (31). We hypothesized that in hTGH, the corresponding FLXL sequence at positions 417–420 could play a role in the interaction of this enzyme with hydrophobic substrates.

We used methods of amino acid deletion and site-directed mutagenesis followed by expression of the mutant proteins in Sf9, Cos-7, and McArdle RH7777 cells and analysis of the lipolytic and esterase activities. Deletion of the entire NLBD resulted in inactivation of hTGH, and based on the lack of solubility of the mutant, it was concluded that the expressed mutant was potentially misfolded. Our NLBD point mutation data show that activity was significantly reduced toward insoluble (lipase) and soluble (esterase) substrates only when F_{417} was replaced by alanine. F_{417} may thus provide an optimum local environment required for efficient substrate hydrolysis. However, our data suggest that F_{417} does not play a significant role in the interfacial activation of the enzyme, because mutation of the residue also decreased hydrolytic activity toward soluble substrates. It is unlikely that the reduced activity in $F_{417}A$ was attributable to misfolding because the mutant was expressed and secreted to similar levels as wt-hTGH, a measure of quality control in the folding of proteins in the endoplasmic reticulum. Carboxylesterases exist as oligomeric and monomeric species, both of which exhibit similar hydrolytic activity (33). Therefore, even if F_{417} were involved in a possible stabilization of the oligomeric form of hTGH, mutation of F_{417} would not be expected to affect the overall hydrolytic activity of the enzyme.

There are five cysteine residues within hTGH based on the deduced amino acid sequence of the cloned cDNA (32). Four of the cysteine residues are involved in intramolecular disulfide bonds, leaving one free cysteine residue (C_{390}). Here, we investigated several possible roles of C_{390}: *i*) intermolecular disulfide bonding *ii*) acylation *iii*) involvement in catalysis. Three enzymatically active forms of a related porcine carboxylesterase (monomer, trimer, and tetramer) were identified by size-exclusion chromatography (53). The natures of the homooligomer interactions were not determined, although all three forms migrated as monomers in reducing SDS-PAGE. It is unlikely that hTGH exists as a covalent dimer in vivo, because purified hTGH from Sf9 and McArdle cells migrates in SDS-PAGE at a "monomeric" molecular mass of 60 kDa in both reducing and nonreducing conditions.

The porcine carboxylesterase was also reported to undergo posttranslational acylation (33). We addressed whether C_{390} in hTGH was acylated, because acylation can affect the subcellular localization of proteins and their association with lipid monolayers (54–56). The substrate pools for TGH are the lipid droplets associated with the endoplasmic reticulum (19). TGH has been shown to coisolate with lipid droplets from liver (17). Acylation could facilitate interaction with the droplet surface. There is no known consensus amino acid sequence for cysteine acylation of proteins therefore, C_{390} of hTGH was a candidate for this modification. However, our data show that C_{390} in hTGH is not acylated and therefore that interaction of hTGH with lipid via this modification is unlikely. These data are also supported by the observations that rat (14, 17) and murine (15) TGH do not have a corresponding free cysteine residue but can hydrolyze the same insoluble substrates.

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