Evidence That Diacylglycerol Acyltransferase 1 (DGAT1) Has Dual Membrane Topology in the Endoplasmic Reticulum of HepG2 Cells

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Triacylglycerol (TAG) synthesis and secretion are important functions of the liver that have major impacts on health, as over-accumulation of TAG within the liver (steatosis) or hypersecretion of TAG within very low density lipoproteins (VLDL) both have deleterious metabolic consequences. Two diacylglycerol acyltransferases (DGATs 1 and 2) can catalyze the final step in the synthesis of TAG from diacylglycerol, which has been suggested to play an important role in the transfer of the glyceride moiety across the endoplasmic reticular membrane for (re)synthesis of TAG on the luminal aspect of the endoplasmic reticular (ER) membrane. This was confirmed by the observation of the loss of both overt (partial) and latent (total) DGAT activity in microsomes prepared from livers of Dgat1−/− mice. Conformational differences between DGAT1 molecules having the different topologies were indicated by the markedly disparate sensitivities of the overt DGAT1 to one of the inhibitors. These data suggest that DGAT1 belongs to the family of oligomeric membrane proteins that adopt a dual membrane topology.

Hypertriglyceridaemia is a key biomarker for the metabolic/insulin resistance syndrome and for associated morbidities, including type-2 diabetes and cardiovascular disease (1). Similarly, excessive accumulation of triglycerides in cytoplasmic lipid droplets results in hepatic steatosis, now recognized as being associated, possibly causatively, with whole body insulin resistance, and which may progress to nonalcoholic fatty liver or steatohepatitis (2, 3). Fasting hypertriglyceridaemia is primarily due to the hypersecretion of triglyceride (TAG) by the liver, within very low density lipoproteins (VLDL). Therefore, an understanding of the enzymology involved in triglyceride synthesis, remodeling, storage, and assembly into secreted VLDL is essential for the design of pharmacological strategies aimed at managing dyslipidaemia without the exacerbation of hepatic steatosis, and vice versa.

Diaclylglycerol acyltransferases (DGATs) catalyze the final reaction of TAG synthesis. Two distinct gene products, DGAT1 and DGAT2, that catalyze most of tissue TAG synthesis have been described (4, 5) but remain relatively poorly characterized, and their respective, nonredundant functions are still to be elucidated. In the present study, we have used two specific inhibitors (which belong to different chemical classes of compounds) of human DGAT1, in combination with the selective permeabilization of the plasma membrane and the ER membrane of whole hepatocytes, to study the sidedness of the active site of the enzyme in situ in HepG2 cells. Our previously described model of hepatic TAG synthesis and secretion (6) was based on observations that (i) only a minority of secreted triglyceride (~20%) is incorporated into VLDL without prior hydrolysis (24) during the co-translational insertion of apoB through the ER membrane to form nascent particles in the first step of lipoprotein assembly (7), (ii) that most newly synthesized TAG is diverted to cytosolic lipid droplets, and (iii) that the hydrolysis of this TAG prior to its assembly into VLDL proceeds only to partial glycerides (8). Equilibration of diacylglycerol across the ER membrane provides the main route through which the acylglycerol moiety is transferred to the secretory pathway. For diglycerides to play this role, DGAT activity would have to be distributed on both aspects of the ER membrane (6, 9, 24) to enable the re-synthesis of TAG on the luminal aspect of the membrane. This was shown to be the case through the demonstration of “overt” and “latent” DGAT activities of approximately equal magnitude in intact microsomes isolated from rat liver (9).

In accordance with this model, we demonstrated that rat liver microsomes express both overt and latent DGAT activities of similar magnitude on the cytosolic and luminal aspects of the ER membrane, respectively (9). We also described how the relative expression of the two activities varies with physiological (11) and nutritional state (4) and responds to treatment of rats with different types of hypolipidemic agent (12). This pathway may also account for the remodeling of TAG that occurs par-
ticularly in the sn-3 position before it is secreted (9). Thus, the positional distribution of acyl chains within TAG molecules differs markedly between intrahepatic and VLDL-TAG, indicating that the extensive hydrolysis-resynthesis cycling between TAG and diacylglycerol serves to remodel TAG before secretion (13). Subsequent data from several other laboratories have supported our model. Thus, the involvement of the ancillary transport of acylcarnitine across the ER membrane for the provision of long-chain acyl-CoA in the ER lumenal compartment necessary for the (re)synthesis of TAG from diacylglycerol delivered from the cytosol across the ER was experimentally established (14–16). In addition, Yamazaki et al. (17) demonstrated that separate adenoviral transfection of the Dgat2 and Dgat1 genes into mouse liver resulted in the accumulation of TAG primarily in the cytosolic and ER-lumenal compartments of hepatocytes, respectively (17).

Recently, overexpression of tagged and mutated murine DGAT1 and DGAT2 has been used to elucidate their membrane topology, in separate studies. The data on DGAT1 (18) suggested that its catalytic site is expressed exclusively on the lumenal aspect of the ER membrane. In the present study we provide evidence that contrasts with this conclusion. We show that the activity due to DGAT1 is expressed on both the cytosolic (overt) and lumenal (latent) aspects of the ER membrane, and that the two activities can be distinguished through differential sensitivity to the two specific inhibitors used. In addition, studies using liver microsomes prepared from Dgat1−/− mice showed that DGAT1 is normally expressed on both aspects of the ER membrane, and also confirmed the results from overexpression studies (see Refs. 19–21) that DGAT2 catalytic activity is exclusively accessible to cytosolic acyl-CoA.

**EXPERIMENTAL PROCEDURES**

**Materials**

Alamethicin, essentially fatty acid-free bovine serum albumin (BSA), d-mannose 6-phosphate, 1,2-dioleoyl-sn-glycerol, oleoyl-coenzyme A lithium salt, lactate dehydrogenase, sodium cacodylate, dithiothreitol, and the lipid standards, glyceryl triacetate, and oleic acid were purchased from Sigma. Digitonin was purchased from Fischer Scientific. Thin-layer chromatography (TLC) plates (LK6D 19 channel) were purchased from VWR Jencons, UK. Tissue culture media were from Invitrogen. All other chemicals used were of analytical grade. Radiolabeled 1-[14C]oleoyl-CoA (50 μCi/μmol) and [3H]glycerol (10 mCi/μmol) were obtained from Amersham Biosciences (GE Healthcare UK Ltd.). The two inhibitors of DGAT activity used were synthesized by Astra Zeneca. The structural formula of inhibitor compound A (iA) is 2-((1s,4s)-4-(4-(4-amino-7,7-dimethyl-7H-pyrimido[4,5-b][1,4]oxazin-6-yl)phenyl)cyclohexyloxy)acetic acid; and B, the structure of compound B (iB), cis-4-[3-fluoro-4-[[5-[(4-fluorophenyl)amino]-1,3,4-oxadiazol-2-yl]carbonyl]amino]phenoxycyclohexanecarboxylic acid.

**Chemical structures of the unrelated compounds used to inhibit human DGAT.** A, the structure of inhibitor compound A (iA), 2-((1s,4s)-4-(4-(4-amino-7,7-dimethyl-7H-pyrimido[4,5-b][1,4]oxazin-6-yl)phenyl)cyclohexyloxy) acid, and B, the structure of compound B (iB), cis-4-[3-fluoro-4-[[5-[(4-fluorophenyl)amino]-1,3,4-oxadiazol-2-yl]carbonyl]amino]phenoxycyclohexanecarboxylic acid.

**Animals**

Male Wistar rats (200–250 g) were from Charles River Laboratories, Essex UK, and Dgat1−/− mice were sourced from Jackson Laboratories. They were fed a standard rodent chow diet. The animals were killed by CO2 anesthesia followed by cervical dislocation.

**Preparation of Liver Microsomes**

The liver was dissected from rats or mice, and immediately chilled in ice-cold homogenization buffer (MI buffer, containing 300 mM sucrose, 5 mM Tris-HCl, 1 mM EDTA, pH 7.4). Homogenization (10–15 strokes) was performed using loose-fitting Potter-Elvenheijm Teflon-glass homogenizers cooled by immersion in ice water. The initial homogenate was centrifuged twice at 2,000 and 12,000 × g to sediment unbroken cells, nuclei, and mitochondria. The postmitochondrial fraction was further centrifuged at 100,000 × g for 60 min at 4 °C to sediment the microsomes. The resulting microsomal pellet was suspended in MI buffer, to ~20 mg of protein/ml, and stored at −80°C in aliquots. The protein concentration was determined by the Bradford method, using BSA as standard.

**Cloning of Human DGAT1 and DGAT2 cDNAs and Expression in Insect Cells**

Insect membranes expressing human recombinant DGAT1 or DGAT2 protein were prepared as in Refs. 4 and 5. The cloning strategies were as follows. For hDGAT1 the plasmid prepared from I.M.A.G.E. clone 412631 containing the coding sequence for human DGAT1 (EMBL accession number AF059202) was used as the PCR template. The primers were: forward, 5’-GGGGAATTCCGGGCTGAGGCCATGGGC-3’ and reverse, 5’-GAGAAGCTTGGCCCT-GACCGCGGC-3’. The PCR was performed using 100 ng of plasmid template DNA, 50 pmol each of the two primers, 200 μM dNTPs, and 0.5 μl of Herculase Hot Start Taq
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polymerase. The resultant PCR product was gel-purified and ligated into the pFASTBAC expression plasmid (Invitrogen) as an EcoRI fragment. The coding sequence was verified, and its correct orientation with respect to the coding promoter was performed through PstI digestion. For hDGAT2 the cloning template was prepared by converting 5 μg of total RNA isolated from the HepG2 cell line into first strand cDNA using the SuperScript Pre-amplification kit (Invitrogen). The resultant cDNA was diluted 1:5, and 1 μl was used as a template in a 50-μl PCR. The PCR primers were: forward, 5'-CGGAATTCCACCC-ATGAAGACCCTCATAG-3' and reverse, 5'-CGGAATTCTT-GGCTAGTCTACCTCCAGGACC-3'. The PCR was performed using 1 μl of cDNA template, 50 pmol each of the forward and reverse primers, 200 μM dNTPs, and 0.5 μl of Hercules Hot Start Taq polymerase. The resultant PCR product was gel-purified and ligated into the pFASTBAC expression plasmid (Invitrogen) as an EcoRI-HindIII fragment. The resultant construct was sequence verified to confirm that the coding region was identical to the sequence quoted in the EMBL accession data base.

Preparation of Baculovirus-transfected Insect Cell Membranes for DGAT Assays

Cell pellets of known weight were re-suspended gently in 10 ml of ice-cold phosphate-buffered saline (PBS) (pH 7.0) and centrifuged twice at 200 × g for 10 min at 4 °C. Pellets were re-suspended in STE buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (disodium salt), pH 7.4), at a ratio of 2 ml of buffer per g wet weight of membranes. The re-suspended pellets were homogenized by 10 passages through a 2-gauge needle using a 10-ml syringe, followed by centrifugation at 100,000 × g (at 4 °C for 60 min). The resultant membrane pellets were re-suspended in STE buffer and the protein concentration was quantified using the Bradford protein assay method.

Hepatocyte Cell Culture

The human hepatoma cell line HepG2, and the rat hepatoma cell line McA-RH7777 were purchased from the EACC. HepG2 cells were cultured routinely in RPMI 1640 medium (with added 2.5 mM L-glutamine) supplemented with 10% fetal bovine serum (FBS). McA-RH7777 cells were cultured in DMEM containing L-glutamine (2.5 mM) and supplemented with 10% FBS. Cells were cultured in a 5% CO2 atmosphere. The cell media were changed every 3–4 days and cells were passaged when they became 80–90% confluencing. The cell media were then changed every 3–4 days and cells were passaged when they became 80–90% confluent, using the same medium containing 2.5 mg of trypsin/ml and 0.1 mM EDTA to detach them.

Permeabilization of HepG2 Cell Plasma and ER Membranes

Aliquots (containing 2 × 106 cells) of trypsin-detached and washed cells were incubated, for known periods of time, with 2 ml of artificial “cytoskeleton” (CSK) medium (23) containing increasing concentrations of digitonin (up to 60 μg/ml as indicated) to permeabilize their plasma membrane, as described in Ref. 23. After the preliminary experiments were performed to find the optimal concentration of digitonin, routine incubations were performed in the presence of 30 μg of digitonin/ml followed, after washing, by incubation with 20 μg of alamethinic/ml, to effect the additional permeabilization of the ER membrane. Total amounts of cytosolic or intra-ER markers were achieved by addition of 1% Triton X-100 to cell suspensions. At the end of the incubation period, the cells were either sedimented and the supernatant used to measure the released cytosolic markers, or used directly to measure the activity of ER-lumenal enzyme activity.

Enzyme Activity and Metabolite Assays

Mannose-6-phosphatase Assay—The assay mixture contained 50 mM sodium cacodylate (pH 6.6), 0.2 mM sucrose, 2.5 mM diithiothreitol, 25 mM mannose 6-phosphate, and 10 mg/ml of defatted BSA. Aliquots (30 μl) of the assay buffer were pre-equilibrated at 37 °C and the assay was initiated by the addition of 125 μl of a suspension containing ~180 μg of cellular or microsomal protein (9). Permeabilized cells that had been previously treated with digitonin (30 μg/ml) to permeabilize the plasma membrane were assayed for mannose-6-phosphatase activity before or after treatment with alamethicin (20 μg/ml in DMSO) aimed at permeabilizing the ER membranes to the substrate of the reaction (mannose 6-phosphate). The reactions were stopped by addition of 125 μl of 20% perchloric acid. The denatured protein was removed by centrifugation, and the amount of inorganic phosphate formed was quantified by addition of an aliquot (200 μl) of the reaction supernatant to 50 μl of inorganic phosphate color reagent (acidic ammonium molybdate-ferrous sulfate mixture), followed by quantification of the absorbance at 700 nm.

Assay of DGAT Activity

Measurement of Overt and Latent DGAT Activity in Permeabilized Cells and in Isolated Microsomes—Routinely, overt DGAT activity of HepG2 and McA-RH7777 cells was measured in situ by carrying out the DGAT assay using cells permeabilized solely by low concentrations of digitonin (30 μg/ml) for 30 min at 0 °C. Latent DGAT activity was measured in HepG2 cells that were further incubated with 20 μg of alamethicin/ml for 30 min at 0 °C. All incubations were for 30 min at 0 °C. Isolated microsomes obtained from rat or mouse livers were used intact (for overt activity) or after permeabilization with alamethicin when total (overt plus latent) DGAT activity was to be measured. The difference between total DGAT activity and overt DGAT activity represented latent DGAT activity. DGAT activity was measured by quantifying the incorporation of [1-14C]-oleoyl-CoA into [14C]triacylglycerol in the presence of the second substrate 1,2-dioleoylglycerol. Preliminary experiments were performed to ascertain that the assay was linear with time and amounts of microsomal and cell protein, and that no DGAT activity was detectable when HepG2 cells were intact. The final assay conditions were as follows: 50 μM [1-14C]-oleoyl-CoA (2.2 × 104 dpm/nmol), 500 μM 1,2-dioleoylglycerol delivered in ethanol (final concentration of ethanol 0.25%), 2.5 mg/ml of BSA, 0.6% DMSO, in 125 mM Tris-HCl buffer (adjusted to pH 7.4 using KOH at 37 °C) containing 10 mM MgCl2 and 250 mM sucrose, and 20 μg of microsomal protein in a volume of 25 μl. Radioactive TAG formation increased linearly for at least the first 5 min, and also had a linear relationship with the amount of protein in the microsomal or permeabilized
cell fractions assayed, respectively. Dioleoylglycerol (added in ethanol, see above) was vortexed into aliquots containing the assay buffer and the microsomal protein, and incubated at 37 °C for 3 min in a shaking heating block to warm the reaction mixture. The reaction was initiated by the addition of the radioactive oleoyl-CoA substrate, and was terminated after 3 min by the addition of chloroform/methanol (2:1, v/v). After vigorous shaking, the chloroform layer was aspirated into a glass tube and dried under a stream of N2 gas. The dried material was re-solubilized in chloroform (200 μl) and the entire volume was applied to TLC plates coated with Silica Gel 60 for separation of the radioactive triglyceride product from the oleoyl-CoA substrate, using hexane/diethyl ether/formic acid (70:30:1, v/v/v) as the mobile phase. A triglyceride standard (triolein, 10 nmol) was used to identify the position of the triglyceride band after lipid separation. Lipid bands were visualized using iodine vapor. The radioactivity of each band was quantified after scraping into scintillation vials, followed by addition of 4 ml of UltimaGold scintillant (PerkinElmer Life Sciences), and quantification of the associated 14C radioactivity using a scintillation counter.

In assays investigating the effect of either of the two hDGAT1 inhibitor compounds (IA and IB) on DGAT activity, these were added in DMSO; the final concentration of the solvent was kept constant at 0.6% of the total assay volume. Incubations of microsomes or cells (previously incubated in the absence/presence of digitonin/alamethacin) with the inhibitors were performed on ice for 5 min prior to starting the DGAT assay. Preliminary experiments on the DMSO tolerance of the assay showed that, at the final concentration (0.6%) of DMSO used, there was no effect on DGAT activity or microsomal membrane integrity. All assays were performed in duplicate on the number of separate preparations indicated in the figure legends.

DGAT Assay in Cells with Permeabilized Plasma Membranes—Confluent HepG2 or Mca-RH7777 cells were detached by trypsinization and incubated on ice in CSK buffer (see above) containing 30 μg of digitonin/ml for 30 min on ice, to expose the overt DGAT activity. They were then sedimented by light centrifugation and resuspended in CSK buffer to wash off the digitonin, followed by gentle sedimentation. They were used either directly (for measurement of overt DGAT activity) or after re-suspension and a further 30-min incubation on ice with CSK buffer containing 20 μg/ml of alamethacin, to expose latent DGAT activity. Alamethacin is a small peptide that forms oligomers that align perpendicularly to the membrane, and in the process, create pores therein.

Assay of the Activities of Recombinant hDGAT1 Expressed in Insect Cell Membranes—Two inhibitors of human DGAT1 activity were used (see “Materials”); they were dissolved separately in DMSO and serial dilutions were dispensed into a 96-well plate in duplicate. Final assay conditions in the reaction mixture were: 40 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.1 mM dicylglycerol, 0.03 mM oleoyl-CoA, and [1-14C]oleoyl-CoA (0.1 μCi/μmol). Final insect membrane protein concentrations were 4.2 μg/ml for DGAT1 and 30 μg/ml for DGAT2 assays, contained in 50 mM Tris-HCl (pH 8.0). Reactions were carried out in 96-well plates for 30 min at 22 °C and initiated by addition of 40 μl of the 1-[14C]oleoyl-CoA-BSA. Assays were terminated by addition of an aliquot of a mixture of 2-propanol and heptane (7:1, v/v) followed by 5 μl of bromphenol blue, 20 μl of carrier lipid mixture (50 μg of triolein and 50 μg of oleic acid in heptane), 100 μl of 0.1 M carbonate buffer (pH 9.5), and 10 μl of [3H]triolein (~10,000 dpm in heptane) as an internal standard, to enable the recovery of extracted triglyceride to be determined. The [14C]triglyceride product formed during the DGAT assay was extracted into the heptane layer, from which a 150-μl aliquot was transferred to a scintillation vial for the quantification of the associated radioactivity.

Measurement of Acyl-CoA:Ethanol O-Acyltransferase Activity—The ethanol used to deliver the dioleoylglycerol (final concentration 5 mM, see above) in the DGAT assay mixture acted as one of the substrates for acyl-CoA:ethanol O-acyltransferase activity (AEAT), thus enabling the simultaneous quantification of AEAT and DGAT activities. Measurement of the activity of this marker enzyme after incubation of cells or membranes in the absence or presence of alamethacin in the same assay as that of DGAT enabled us to determine, simultaneously, the degree of intactness of the individual microsomal membrane preparations, as AEAT is known to be exclusively latent in its ER distribution (24). The very low activities of AEAT detected in microsomes and digitonin-treated cells not exposed to alamethacin treatment indicated that both isolated microsomes and those left in situ in permeabilized hepatocytes were very largely intact.

Quantification of Triglyceride Synthesis and Secretion by HepG2 Cells—Cells (1.5 × 106) were cultured for 72 h in 10-cm2 six-well dishes in 2 ml of RPMI 1640 medium (with added l-glutamine, 2.5 mM) supplemented with 10% FBS. They were subsequently washed in PBS, and preincubated in serum-free medium for 30 min with increasing concentrations of the inhibitors (IA or IB) dissolved in DMSO. The control reactions had only DMSO added (0.6% DMSO in all incubations). At the end of the incubation period, oleate (0.7 mM) and [3H]glycerol (0.25 mM, 104 dpm/nmol) were added to each well and cells were incubated at 37 °C for 2 h (which was determined from preliminary experiments to be on the linear time course of incorporation of [3H]glycerol into triglycerides). At the end of the 2-h incubation period, total lipids were extracted from the media and the cells using chloroform:methanol (2:1, v/v). The triglyceride fraction was isolated, and the radioactivity associated with it quantified, as described above for the DGAT assays.

Glycerol 3-Phosphate and Lactate Dehydrogenase Assays—When plasma membrane integrity was measured, cells were incubated with 20 mM glycerol for 20 min prior to treatment with different concentrations of digitonin. Following sedimentation of the permeabilized cells by light centrifugation, the incubation media were assayed for glycerol 3-phosphate content. The glycerol 3-phosphate assay mixture contained 0.76 M hydrazine sulfate, 2 mM glycine, and 10.5 mM EGTA (pH 9.0) in 250 μl, to which was added to 50 μl of NAD (40 μM), followed by 700 μl of the permeabilized cell supernatant. The optical density at 340 nm was monitored using a spectrophotometer, and the reaction was initiated by the addition of 5 μl of an ammonium sulfate suspension of purified glycerol-3-phosphate dehydrogenase (Sigma). The optical density was followed continuously to the completion of the reaction, and the amount
of glycerol 3-phosphate present was calculated from the difference between the initial and final optical density at 340 nm. Lactate dehydrogenase activity was measured spectrophotometrically by monitoring the NADH-dependent absorbance changes at 340 nm.

**Statistical Analysis**

The data presented are mean (± S.E.) of the number of separate preparations detailed in the figure legends. Statistical significance was calculated using Student’s t test.

**RESULTS**

**Overt and Latent DGAT Activities in Microsomes and Permeabilized Hepatocytes**—To ascertain whether the previous observation of the dual distribution of DGAT activity in isolated microsomes (9) could be reproduced in the ER in situ after permeabilization of whole cells, we measured DGAT activity before and after treatment with the pore-forming peptide, alamethacin, both in rat-derived McA-RH7777 cells and human-derived HepG2 cells in which the plasma membrane was permeabilized with low concentrations of digitonin (see below), and in isolated rat microsomes. The data in Fig. 2, panel i, show that permeabilization of isolated rat-derived liver microsomes with alamethacin (a pore-forming small peptide) resulted in the expected increase in DGAT activity (cf. Ref. 9) and ~10-fold increases in the exposure of the activities of AEAT (Fig. 2, panel ii) and mannose-6-phosphatase (Fig. 2, panel iii).

Prior to routine use of McA-RH7777 and HepG2 cells, preliminary experiments were performed to ascertain the concentrations of digitonin required to permeabilize selectively the plasma membrane of the cells, without affecting membrane integrity of the ER (Fig. 3). The data show that optimal release of a low molecular weight metabolite, glycerol 3-phosphate, was achieved at digitonin concentrations (30 μg of digitonin/ml) that had minimal effects on the exposure of mannose-6-phosphatase activity, a nonreleasable marker for the latent aspect of the ER membrane (Fig. 3). Release of the soluble, cytosolic enzyme lactate dehydrogenase (molecular mass ~140 kDa) by digitonin occurred much less readily (Fig. 3) indicating that disruption of the plasma membrane by these low concentrations of digitonin discriminated in favor of small molecules (cf. Ref. 25). Importantly, the additional incubation of cells with alamethacin (20 μg/ml), which we used after digitonin to permeabilize the ER, did not increase the amount of glycerol 3-phosphate released by a maximally effective concentration (30 μg/ml) of digitonin alone (not shown), indicating that the ability of alamethacin to expose mannose-6-phosphatase activity was entirely due to ER permeabilization and not any additional effect it might have on the integrity of the plasma membrane. Therefore, we routinely used 30 μg of digitonin/ml to obtain cells in which the plasma membrane was permeabilized to oleoyl-CoA, and successive incubations with 30 μg of digitonin/ml followed by 20 μg of alamethacin/ml further to make the luminal aspect of the ER membrane accessible to oleoyl-CoA. This enabled us to measure overt and total (overt plus latent) DGAT activities, respectively.

There was a significant increase (an approximate doubling) in DGAT activity when the ER of cultured McA-RH7777 and HepG2 cells was permeabilized with alamethacin (Figs 4, panels i and ii, respectively). The ratio of total to overt DGAT activity in rat-derived McA-RH7777 cells was very similar to that obtained with isolated rat liver microsomes (compare Figs. 2 and 4, panel i), indicating that the conclusions drawn about the dual localization of DGAT activity are valid also in the context of the ER in situ in whole cells. Moreover, the data in Fig. 4, panel ii, show that the dual distribution of DGAT activity on either aspect of the ER membrane is also present in HepG2 cells.

**Inhibitors of Human DGAT1**—Two chemically unrelated inhibitors of human DGAT1 (iA and iB, see “Experimental Procedures”).
procedures” and Fig. 1 for chemical and structural formula) were tested for efficacy and specificity using the enzyme protein expressed in insect cells, which were used as a source of either hDGAT1 or hDGAT2, to test for inhibitor specificity. Fig. 5 shows dose-response curves for iA and iB, which were shown to be specific to the inhibition of hDGAT1 with IC50 values of 38.3 and 3.4 nM, respectively (Fig. 5, panels i and ii). Both compounds were found to be noncompetitive inhibitors of hDGAT1 (data not shown). Both compounds were inactive against hDGAT2 at >100-fold higher concentrations than those sufficient to inhibit 95% of hDGAT1 activity (Fig. 5). Therefore, both iA and iB were considered to be specific for hDGAT1 inhibition at the nanomolar concentrations used in subsequent experiments (see below). Neither of the two compounds showed any inhibitory activity against human MGAT1 similarly expressed in insect cells (not shown).

As expected from the fact that both inhibitors were developed against human DGAT1, the IC50 values of these compounds were much higher when they were tested on DGAT activities in either rat or mouse liver microsomes (not shown). Therefore, subsequent experiments on the use of these inhibitors on the ER in situ (in permeabilized cells) were conducted exclusively on a human-derived cell line, HepG2.

Inhibition of Overt DGAT Activities in the ER of Permeabilized HepG2 Cells—HepG2 cells were treated either with 30 μg of digitonin/ml alone, to permeabilize the plasma membrane and measure overt DGAT activity, or additionally with 20 μg of alamethacin/ml, to measure total (overt plus latent) DGAT activity of the ER in situ, in the absence or presence of either iA (1.5 μM) or iB (240 nM) at the respective concentrations at which they were maximally effective against hDGAT1 expressed in insect cells. In preliminary experiments, we tested whether the inhibitors were cell permeable. HepG2 cells were incubated with iA or iB either when they were intact, or permeabilized with digitonin alone or additionally with alamethacin. As shown in Fig. 6, panel i, both iA and iB inhibited TAG synthesis and secretion in intact cells. In preliminary experiments, we tested whether the inhibitors were cell permeable. HepG2 cells were incubated with iA or iB, either intact, or permeabilized with digitonin alone or additionally with alamethacin. As shown in Fig. 6, panel ii, neither of the two inhib-
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FIGURE 5. Dose-response curves for the inhibition of human DGAT1 and DGAT2 expressed in insect cell membranes. The human DGATs (hDGAT1 and hDGAT2) were expressed in insect cells using baculovirus (see “Experimental Procedures”). The effects of increasing concentrations of inhibitors iA and iB (panels i and ii, respectively) on the DGAT1 (●) and DGAT2 (□) activity associated with the membranes were quantified. Values are mean ± S.E. Dose-response curves and IC50 values were generated from three separate determinations, and IC50 values were computed using Origin software and were 38.3 and 3.4 nM for iA and iB, respectively.

Inhibitory activity of the two compounds on overt and latent DGAT activity was measured at their maximally effective concentrations (derived from the data in Fig. 5). As shown in Fig. 7, panel i, iB (24 nM) inhibited ~60% of overt DGAT activity and 100% of the latent activity. These observations showed that DGAT1 activity is expressed on both the overt and latent aspects of the ER membrane and that both are inhibitable by iB. By contrast, 150 nM iA, a concentration that inhibited hDGAT1 fully in insect cell membranes, had no effect on overt DGAT activity in the ER of HepG2 cells, whereas it inhibited 100% of the latent DGAT activity (Fig. 7, panel i).

These observations suggested (a) that DGAT1 activity is expressed on both sides of the ER membrane (effects of iB), and (b) that at concentrations of the two inhibitors that are maximally effective on hDGAT1 expressed in insect cell membranes, only latent DGAT1 of HepG2 cells is sensitive to inhibition by iA, whereas both overt and latent DGAT1 are sensitive to inhibition by iB.

Differential Sensitivity of Overt and Latent DGAT1 Activities to Compounds iA and iB—To test whether this inference was valid, we performed dose-response studies for both inhibitors on overt and latent DGAT activities in HepG2 cells. As shown in Fig. 7 (panels ii and iii), the IC50 values for iB acting on overt DGAT1 was the same as that for latent DGAT1 (Fig. 7, panel iii) and for the effects of this inhibitor on hDGAT1 expressed in insect cells (see Fig. 5, panel ii). By contrast the IC50 for iA inhibition of overt DGAT1 was >15-fold higher than the IC50 for its effect on latent DGAT activity in HepG2 cells (Fig. 7, panel ii) and on hDGAT1 expressed in insect cell membranes (Fig. 5, panel i). This differential sensitivity of overt and latent DGAT1, when present in the ER membrane in situ, to iA (but not to iB) indicated that the two populations of DGAT1 molecules that result in overt and latent activities have conformations and/or interactions with other components of the ER membrane that make them molecularly distinguishable to compound iA. Importantly, we measured AEAT activity contemporaneously with each individual DGAT assay, and therefore could establish that the inhibition of overt DGAT activity only at higher concentrations for iA could not have been due to an artifactual effect of this compound on the integrity of the membrane and exposure of latent DGAT activity. AEAT activities remained at background levels even at the highest concentrations of iA used (see also Fig. 6, panel ii).

Overt and Latent DGAT Activities in Liver Microsomes Prepared from Dgat1−/− Mice—To determine whether disruption of the Dgat1 gene results in an altered distribution of DGAT activity in the ER, liver microsomes were prepared from wild-type and Dgat1−/− mice (same genetic background). The results in Fig. 8 show that disruption of the Dgat1 gene affects the DGAT activity present on both aspects of the ER membrane. Thus, latent DGAT activity was totally abolished, whereas overt DGAT activity was significantly diminished (by ~35%) compared with wild-type mouse liver microsomes. These results could not be explained by altered ER membrane permeabilization by alamethacin, as AEAT activity was exposed equally by this treatment in microsomes isolated from both wild-type and Dgat1−/− mouse livers (not shown). These data confirm for the mouse that (i) DGAT1 activity accounts for all the latent activity of DGAT in the ER, and that (ii) DGAT1 constitutes a substantial proportion of the overt DGAT activity of these membranes, although overt DGAT1 activity constitutes a smaller proportion of overall cytosol-facing DGAT activity in mouse ER compared with that in human-derived HepG2 cells.

DISCUSSION

The currently accepted model for the role of diglyceride in the synthesis of 80% of (remodeled) triglyceride destined for secretion within VLDL has been described and discussed in detail elsewhere (see Introduction). The present data validates and adds significantly to key aspects of the model (6).

In the present study we have shown (Fig. 2) that dual DGAT activities can also be detected on both sides of the barrier to
acyl-CoA represented by the ER membrane when the ER is left undisturbed in situ, both in human- and rat-derived hepatoma cells (HepG2 and McA-RH7777 cells, respectively). Although it is to be appreciated that when the ER membrane is left intact, DGAT activity on the cytosolic aspect of the ER membrane itself as well as that on lipid droplets and mitochondria (see Refs. 19–21) will also be accessible to the acyl-CoA substrate, the detection of DGAT activity on both sides of the ER membrane is significant, as the detection of dual localization in isolated microsomes (which have to re-seal “right side-out” into vesicles when the planar sheets of the ER are disrupted during tissue homogenization) depends on the method of preparation of this subcellular fraction. Membrane intactness is not invariably achieved by different laboratories, and depends on the method of tissue/cell disruption. By contrast, in whole cells, the preferential permeabilization of the plasma membrane is reproducibly achieved using low concentrations of digitonin, by virtue of the much higher content of cholesterol in this membrane than in the ER (25). The subsequent use of a second membrane-permeabilizing agent, namely alamethacin, a pore-forming peptide that acts through an entirely different membrane permeabilization mechanism, allowed the discrete quantification of overt and latent DGAT activities in situ in cells without disruption of the native ER structure. Importantly, the similarity of the ratios of latent to overt DGAT activities as quantified in whole (rat-derived) McA-RH7777 cells and in rat isolated liver microsomes suggests that our original observations (9, 11, 12, 26) are unlikely to have been due to artifacts arising during the preparation of liver microsomal fractions.

The current data show that DGAT1-mediated activity is present on both the cytosolic and luminal aspects of the ER membrane in whole hepatocytes. This conclusion is drawn from two independent approaches. First, iB, a highly specific inhibitor of DGAT1 (Fig. 5, panel iii), inhibits a large proportion (~60%) of overt DGAT activity in HepG2 cells (Fig. 7, panel i). This indicates that in HepG2 cells the activity due to DGAT1 represents the majority of overt DGAT, with the remainder presumed to be contributed by DGAT2 (which also resides on lipid droplets and the mitochondria (see Refs. 19–21) and MGAT1 (both of which were shown not to be inhibited by either of the two inhibitors even at 100-fold higher concentrations than used routinely). Second, in liver microsomes isolated from wild-type mice, there was the expected increase (approximate doubling) in DGAT activity upon treat-
ment with alamethacin, indicating that in mouse liver ER also the same pattern of overt and latent DGAT activities occurs as in rat liver microsomes and in the ER of HepG2 and McA-RH777 cells. By contrast, in liver microsomes prepared from $Dgat1^{-/-}$ mice, there was no difference between the activities of DGAT in intact and alamethacin-permeabilized microsomes (Fig. 8, see also our preliminary data cited in Ref. 19) indicating that all latent DGAT activity was lost upon disruption of the $Dgat1$ gene. Moreover, a significant proportion (~35%) of overt DGAT activity of isolated microsomes was also lost in $Dgat1^{-/-}$ mice (Fig. 8), indicating that DGAT1 is also expressed on the overt aspect of the ER membrane in mouse liver microsomes too. The absence of any latent DGAT activity in the microsomes of $Dgat1^{-/-}$ mice (Fig. 8) also shows that DGAT2 makes no contribution to latent DGAT activity in mouse liver, and that the DGAT2 active site is exclusively exposed on the cytosolic aspect of the ER membrane and other structures exposed to the cytosolic compartment (see Refs. 19–21).

The present conclusion that DGAT1 has dual topologies within the ER is at variance with the recent conclusion (18) drawn from experiments in which FLAG- and Myc-tagged and mutated constructs of murine $Dgat1$ cDNA were overexpressed in HEK-293T cells. The authors of that study concluded that the active site of DGAT1 is expressed exclusively on the lumen-facing aspect of the ER membrane (18). It is not clear why this single topology for DGAT1 was obtained in those studies. It is possible that overexpression per se, or the introduction of tags into the mutated proteins (27), or the use of heterologous expression (murine cDNA in a human embryonic kidney cell-line) may have precluded the proper regulation of the insertion of the resulting proteins into the ER membrane because of the lack of the appropriate molecular machinery (e.g. chaperones/accessory proteins (28), protein kinases/phosphatases) required to effect dual topology of the DGAT1 protein (see below). In this respect, it is of interest that for the similarly heterologously expressed hDGAT1 in insect cell membranes we detected no evidence of a dual sensitivity to iA; the dose-response curves for both iA and iB were entirely monophasic and each could be fitted by a single sigmoid equation (Fig. 5).

The present approach of studying the native, endogenously expressed enzyme avoids such potential complications. Indeed, our findings on the dual topological expression of DGAT1 activity are in agreement with previous observations made in studies of the homologous hepatic expression of native, full-length murine DGAT1 and DGAT2 proteins in the liver of mice. Thus, Yamazaki et al. (17) found that hepatic expression of DGAT1 in mice through adenovirus-mediated gene trans-
Dual Topology of DGAT1 in Hepatocyte ER Membrane

In situ differential sensitivity of overt and latent DGAT1 activities of liver microsomes isolated from treated animals. By contrast, transfection with DGAT2 resulted in the elevation only of overt DGAT activity (17).

The differential sensitivity of overt and latent DGAT1 activities to iA (but not to iB) suggests that the two distinct topologies of DGAT1 in the ER membrane in situ that result in overt and latent activities may affect the conformation of the inhibitor-binding site and/or its interaction with other membrane components so as to decrease the affinity of those molecules with their active site exposed on the cytosolic aspect of the ER membrane for iA, but not that for iB.

The occurrence of dual topologies for membrane proteins is a well-established phenomenon (29), and is increasingly recognized as an important property of oligomeric membrane proteins in particular (30) in which it is achieved when individual oligomers (including those generated from the same gene, i.e. “singletons” (30, 31)) insert in opposite orientations in membranes, thus resulting in their active sites being exposed on both sides of the membrane simultaneously (10, 27, 28, 30–33). It may be relevant that DGAT1 is an oligomeric membrane protein, and appears to exist in dimeric and tetrameric forms (18). Proteins with dual topology are known to have weak signals (net differences in charged amino acids next to membrane boundary regions of the protein sequence so as to control transmembrane domains (30). Interestingly, for at least one known bacterial protein, the relative proportions of the opposing topological orientations depend on growth conditions (10, 33), raising the possibilities that (i) the ratio between the two orientations may not always be unity, and (ii) post/co-translational modification(s) may alter the net charges at critical membrane boundary regions of the protein sequence so as to control the relative proportions of the two alternative orientations depending on the prevailing activity of protein modifying enzymes. If these considerations can be extrapolated to DGAT1, they may explain our previous observations that the relative amounts of overt and latent DGAT activity in the ER of rat liver (which will be dependent on the relative distribution of DGAT1 activity as this accounts for the majority of the overt DGAT activity) are capable of modulation by physiological state and by hypolipidemic drug treatment (6).

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