RESEARCH COMMUNICATION

Overt and latent activities of diacylglycerol acytransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion

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The possibility that triacylglycerol (TAG) synthesis occurs on both aspects of the endoplasmic-reticular membrane during the process of incorporation of TAG into secreted very-low-density lipoprotein (VLDL) [Zammit (1996) Biochem. J. **314**, 1–14] was investigated by measuring the latency of diacylglycerol acyltransferase (DGAT) in microsomal fractions obtained from rat liver homogenates. Permeabilization of microsomes with taurocholate resulted in the doubling of the activity, indicating that DGAT activities of approximately equal magnitude occur on either aspect of the microsomal membrane. The taurocholate concentrations required for exposure of the latent activity of DGAT were identical with those that resulted in the exposure of marker enzymes for the lumen of the endoplasmic reticulum. Fractionation of the microsomes into smooth and rough populations indicated that the distribution of overt and latent DGAT activities was the same throughout. The possibility that taurocholate effects may result from non-specific activation of the overt enzyme was excluded by employing the channel-forming peptide alamethicin to effect permeabilization, and by varying the mode of delivery of diacylglycerol substrate to the microsomal membranes. Permeabilization using alamethicin gave a slightly higher latent/overt ratio for DGAT. The possible roles of overt and latent DGAT activities in the synthesis and secretion of TAG by the liver are discussed.

INTRODUCTION

The mechanism of the incorporation of triacylglycerol (TAG) into secreted very-low-density lipoprotein (VLDL) in the liver has attracted considerable interest (for reviews see [1–3]) because of the potential for pharmacological intervention in the modulation of hepatic TAG secretion. The work of several laboratories [4–6] has indicated that a two-step mechanism is involved. Thus nascent particles are relatively lipid-poor and have a density similar to that of high-density lipoprotein or low-density lipoprotein. The addition of further TAG to the lipid core is proposed to occur through the fusion of nascent apolipoprotein B (apoB)-containing particles with TAG droplets that exist independently within the lumen of the smooth endoplasmic reticulum (SER) and are not associated with apoB [7]. A precursor–product relationship between the denser nascent particles and the secreted VLDL has most clearly been demonstrated for $apoB_{48}$-containing VLDL in McARH7777 rat hepatoma cells [6], but it may also hold for $apoB_{100}$-containing particles as shown by the studies of Swift [5] and from the fact that the apparent inability of HepG2 cells to perform the second step results in the secretion of only lipid-poor apoB-containing particles [8].

It is well established that cytosolic droplet TAG cannot be incorporated *en bloc* into VLDL [1,3,9,10]. The work of Yang et al. [11] showed that there are important differences between the stereospecific distribution of the fatty acyl moeities in intrahepatic TAG and in VLDL TAG. These differences suggest that the route from cytosolic droplet TAG to VLDL TAG involves hydrolysis to 1,2-diacyl-*sn*-glycerol (DAG), followed by remodelling of the DAG fatty acyl composition through trans-esterification, and the re-esterification of the remodelled DAG to TAG, before secretion (see [3]). This conclusion was supported by data from experiments *in vivo* [12], in which the incorporation of fatty acids synthesized *de novo* from 6-[^{2}H]ethanol into liver TAG and VLDL TAG was monitored. In [11] it was suggested that re-esterification of DAG occurs on the cytosolic aspect of the endoplasmic reticulum (ER) membrane because the membrane is impermeable to acyl-CoA esters, and diacylglycerol acyltransferase (DGAT) activity has been repeatedly suggested to be located exclusively on the cytosolic face of the membrane [12–15]. The first of these apparent obstacles for intraluminal DAG esterification to TAG appears to have been resolved by the recent observations [16,17] that microsomes contain overt and latent forms of carnitine acyltransferases. This suggests that, by analogy with the mitochondrial inner-membrane system, an acylcarnitine translocase could exist to mediate the transfer of acyl moeities (as acylcarnitines) across the ER membrane. With regard to the second obstacle, we have recently pointed out [3] that, contrary to the generally accepted view [12–15], the data purporting to show that DGAT is located exclusively on the outer face of the microsomal membrane were inconclusive, and we suggested that esterification of DAG to TAG may occur on both sides of the ER membrane. Therefore, in the present study, we have re-evaluated the distribution of DGAT activity on both sides of the ER membrane to test the possibility that both overt and latent activities may exist.

MATERIALS AND METHODS

Animals

Male Wistar rats (350–450 g) were kept in controlled light (12 h light/12 h dark) conditions and maintained on standard lab-

Abbreviations used: apoB, apolipoprotein B; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; TAG, triacylglycerol; VLDL, very-low-density lipoprotein.

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oratory chow (see [18]). The animals were starved for 24 h before being killed at 1 h into the light period.

Microsomal preparation

Three livers were homogenized in ice-cold medium containing 300 mM sucrose/1 mM EGTA/5 mM Tris/HCl, pH 7.4, and microsomes were prepared from the post-mitochondrial (10000 ***g***) fraction by centrifugation at 100000 ***g*** for 70 min at 4 °C. The resultant pellets were resuspended to a final volume of 10 ml, separated into aliquots, which were snap-frozen in liquid N_2 and stored at −80 °C. SER and rough endoplasmic reticulum (RER) vesicles were prepared from freshly prepared, crude microsomes by separation on a linear sucrose density gradient. The gradient was prepared by mixing the appropriate proportions of 1.80 M and 0.37 M sucrose (in 5 mM Tris, pH 7.4) to a total volume of 8 ml. The microsomal suspension (3.5 ml) was layered on to the gradient, contained in 13 ml tubes, and centrifugation was performed at 100000 ***g*** for 70 min at 4 °C in a Sorvall TH641 swing-out rotor. Twelve 1 ml fractions were collected from the gradient by displacement with a solution of 2.3 M sucrose. The fractions were diluted with water to give a final concentration of 0.25 M sucrose, frozen in liquid N_2 and stored at −80 °C until required. Preliminary experiments established that a single cycle of freezing and thawing (either at 0 °C for 60 min or at 22 °C for 5 min) did not alter the absolute activity or the overt/latent ratio of DGAT or of any of the marker enzymes studied, when compared with the values obtained with freshly prepared microsomes. In some experiments SER vescicles were prepared by the method of [19]. Briefly, post-mitochondrial supernatants (9.85 ml) were added to 0.15 ml of 0.1 M CsCl. The resulting solution was layered on to a cushion (15 ml) of 1.3 M sucrose/15 mM CsCl/1 mM EGTA/5 mM Tris/HCl, pH 7.4. SER vescicles were collected from the 0.3 M–1.3 M sucrose interface after centrifugation at 87000 ***g*** for 160 min at 4 °C.

Permeabilization of microsomal vescicles with taurocholate was performed as described previously [20,21]. Incubations were, on ice, for 30 min at the taurocholate concentrations indicated. The protein concentration was kept at 1.5 mg/ml. Permeabilization with alamethicin was performed under the same conditions, with the peptide added as a concentrated (80 mg/ml) ethanol solution. The final concentrations were 3 mg of protein/ml, 0.06 mg of alamethicin/ml and 0.38% (v/v) ethanol. Preliminary experiments showed that, at the final concentration used, ethanol did not affect DGAT activity or microsomal membrane integrity (cf. [22]).

Protease treatment

Microsomes (3 mg of protein/ml) were treated with each of the following proteases; trypsin, chymotrypsin, subtilisin, papain and proteinase K. The concentration of protease was varied from 10 to 400 μg/ml and incubations, on ice, were for up to 60 min. Where appropriate, soya-bean trypsin inhibitor (4.16 mg/ml) and 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride (1.66 mM) were added together with albumin (1 mg/ml final concentration) to terminate the protease action. The membranes were sedimented by centrifugation at 100000 ***g*** for 60 min at 4 °C and resuspended in assay buffer before being added to the reaction mixture. Assays on control samples, treated similarly but without proteases, were run concurrently.

Enzyme assays

DGAT and acyl-CoA:ethanol acyltransferase activities were measured simultaneously in a medium of 300 mM sucrose/10 mM Tris/HCl/10 mM $MgCl_2$/0.8 mM EGTA containing 1 mg of defatted BSA and 120 μM [^{14}C]palmitoyl-CoA (22000 d.p.m./nmol). In order to test whether the mode of delivery of diacylglycerol substrate to the membranes affected the results we used two substrates. The first was 1 mM 1,2-dioleoyl-*sn*-glycerol (added to the assay mixture as a concentrated solution in ethanol; the final ethanol concentration was 0.1% of total volume [15]). The container holding the assay mixture was flushed with N_2 gas, sealed and sonicated in a sonicator bath at 40 ° for 2 min. Just before the assay, aliquots (200 μl) of the mixture were transferred to 1.5 ml plastic tubes and equilibrated to 37 °C in a shaking heating block, the assays were started by the addition of microsomes. The second diacylglycerol substrate used was dipalmitoylglycerol, delivered as a sonicated mixture with phosphatidylglycerol, prepared as described previously [23]. Briefly, assay buffer (see above) was added to the lipids to give nominal concentrations of 3.33 mM and 2.67 mM respectively. The mixture was warmed to 65 °C and sonicated using a 2.5 mm-microprobe sonicator (Kontes, Burkard Scientific, Uxbridge, Middx., U.K.) operated at 20 μm and at 80% of maximal power. Sonication was performed under a stream of N_2 for 20 periods of 15 s, over 40 min. The opaque but homogeneous mixture was then diluted with the final assay mixture to give lipid concentrations of 1 mM dipalmitoylglycerol and 0.8 mM phosphatidylglycerol. The complete assay mixture was sonicated for 15 s at 37 °C, before separation into assay tubes. Ethanol (0.7%) was included to enable the activity of acyl-CoA:ethanol acyltransferase activity to be measured. When preincubations with alamethicin were performed, the amount of ethanol carried over into the assay medium was allowed for. All assays were initiated by the addition of microsomal protein (75 μg of protein in a volume of 50 μl) and were terminated after 4 min by the addition of 0.75 ml of chloroform/methanol (2:1, v/v) containing 20 μg/ml of trioleoylglycerol as carrier. The chloroform-extractable material was separated by TLC on silica-gel 60 analytical plates using hexane/diethyl ether (4:1, v/v). Radioactivity associated with triacylglycerol, palmitate and ethyl palmitoyl ester was quantified using a PhosphorImager (Molecular Dynamics). A range of standards containing known amounts of ^{14}C were applied to the plate, to allow absolute quantification of the radioactivity in the experimental samples.

Mannose-6-phosphatase activity was measured essentially as described in [22]. The assay buffer was 50 mM sodium cacodylate (pH 6.6)/0.2 M sucrose/2.5 mM dithiothreitol/25 mM mannose 6-phosphate containing 10 mg/ml of defatted BSA. Aliquots (30 μl) were pre-equilibrated to 37 °C and assays were initiated by the addition of prewarmed microsomal suspension (125 μl, containing 180 μg of protein). The reaction was terminated after 10 min by the addition of 125 μl of 20% (v/v) perchloric acid. P_i was quantified in the supernatant after the sedimentation of denatured protein.

Glycerol 3-phosphate acyltransferase activity was measured as described previously [24]. NADPH:cytochrome *c* oxidoreductase was measured as described in [25], and RNA as in [26]. P_i and protein were measured according to the manufacturers' instructions (see below).

Materials

1-[^{14}C]Palmitoyl-CoA and glycerol 1-[^{14}C]trioleoylglycerol were obtained from Dupont NEN Research Products. Silica-gel 60 TLC plates were from Merck. Palmitoyl-CoA (potassium salt) was from Pharmacia Biotech and proteases, 1,2-dioleoylglycerol, alamethicin, dipalmitoylglycerol and phosphatidylglycerol were from Sigma (Poole, Dorset, U.K.). P_i and protein-determination

kits were from Sigma and Bio-Rad (Hemel Hempstead, Herts., U.K.) respectively.

RESULTS AND DISCUSSION

Latency of DGAT activity in total microsomal fractions

The optimal palmitoyl-CoA/albumin concentration ratio that would allow assay of DGAT activity in both intact and permeabilized microsomes was established to be 100 μM palmitoyl-CoA in the presence of 12 mg of albumin/ml, in spite of the several-fold (4.1 ± 0.5, $n = 3$) greater rate of hydrolysis of palmitoyl-CoA in the permeabilized microsomes. In these experiments, total microsomal fractions prepared from rat liver homogenates were used. The activity of DGAT was measured in parallel with the activity of two enzymes (mannose-6-phosphatase and acyl-CoA: ethanol acyltransferase), well-established as being fully latent in intact or lysed [with 0.6 % (w/v) taurocholate] microsomes. The results (means $\pm$ S.E.M.) indicated that 46.7 ± 6.5 % ($n = 9$) of total DGAT activity was latent.

Distribution and latency of DGAT in SER and RER

The presence of overt and latent DGAT activity in microsomes raised the question as to whether this dual membrane localization is related to the two peaks of DGAT activity observed in SER and RER, respectively, of microsomes prepared from HepG2 cells [27]. In order to establish if bimodal distribution is also a feature of rat liver (which, in contrast with HepG2 cells, can perform both steps of TAG incorporation into VLDL [8]), we measured DGAT activity in intact and taurocholate-treated SER- and RER-enriched microsomal populations. Figure 1 shows that, although rat liver resembles HepG2 cells in having a bimodal distribution of total DGAT, it differs in that the activity is enriched in the RER rather than in the SER [27].

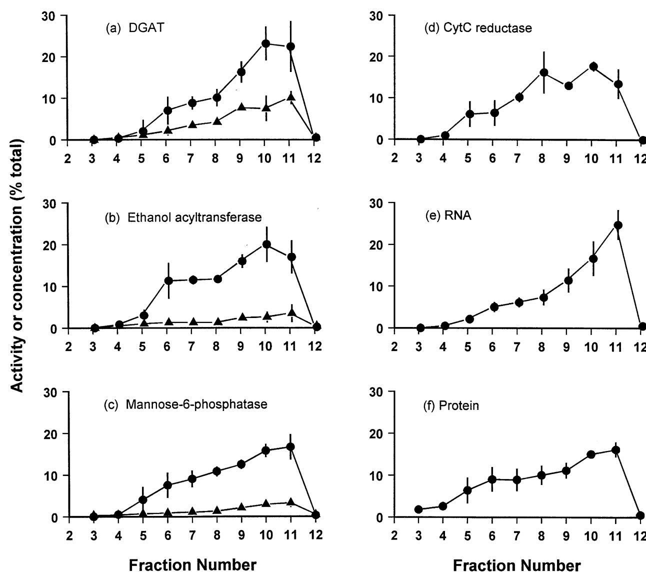


Figure 1 Distribution of DGAT and marker enzyme activities in microsomes subjected to sucrose-density-gradient fractionation

The overt activities of DGAT and marker enzymes in the microsomal fractions before permeabilization (▲) and after permeabilization (overt plus latent) (●) are shown in (**a**)–(**c**). Microsomal suspensions were layered on to a continuous sucrose density gradient (0.367 M–1.796 M) and centrifuged at 100000 ***g*** for 70 min. Fractions were collected by vertical displacement and the activities of (**a**) DGAT, (**b**) acyl-CoA: ethanol acyltransferase and (**c**) mannose-6-phosphatase were measured before and after taurocholate treatment. (**d**) NADPH: cytochrome *c* oxidoreductase (CytC reductase) activity, (**e**) RNA and (**f**) protein content were also measured. The values represent the total activities or amounts recovered in each fraction as a percentage of those in the unfractionated (crude) microsomal suspension, and are expressed as means ($\pm$ S.E.M.) of three gradient fractionations of separate microsomal preparations. The absolute activities of DGAT in intact (overt) and taurocholate lysed (total) crude microsomes were 0.38 ± 0.13 and 0.70 ± 0.20 nmol/min per mg of protein respectively. The total activities of acyl-CoA: ethanol acyltransferase, mannose-6-phosphatase and NADPH: cytochrome *c* oxidoreductase were 0.46 ± 0.18 μmol/min, 56.2 ± 8.9 nmol/min and 0.14 ± 0.03 nmol/min per mg of protein at 37 °C respectively.

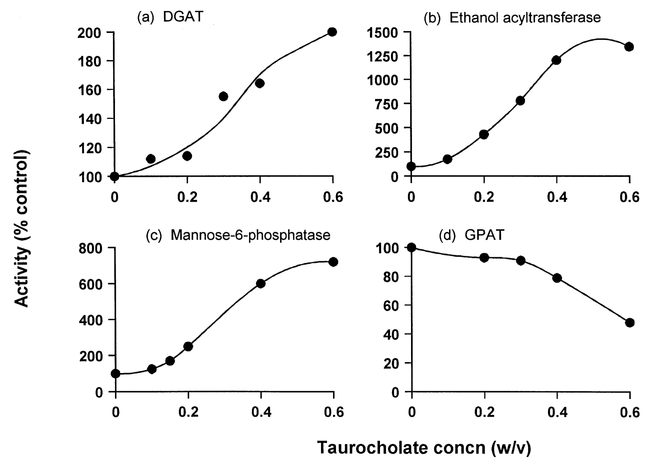


Figure 2 Effects of increasing taurocholate concentration on the activity of (a) DGAT, (b) mannose-6-phosphatase, (c) acyl-CoA: ethanol acyltransferase and (d) glycerol 3-phosphate acyltransferase (GPAT) in rat liver microsomes

The values are from a representative experiment repeated three times.

In [27], because the microsomal fractions were solubilized with taurocholate before the enzyme assay, the latency of DGAT in HepG2 cells was not studied. The data in Figure 2 indicate that the degree of latency of DGAT activity did not vary appreciably between the SER- and RER-enriched fractions. This observation appears to discount the possibility that the lumen-facing DGAT II is located preferentially in the SER, where the formation of non-apoB-associated neutral lipid droplets is thought to occur [7].

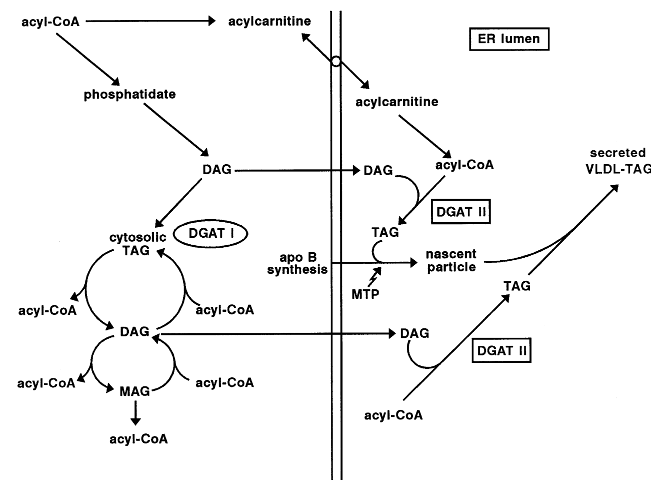
Validation of permeabilization effects

In order to ascertain that the apparent loss of partial latency of DGAT in rat liver microsomes was due to permeabilization of the membranes rather than a non-specific protein–detergent effect on DGAT activity (e.g. activation of a cytosol-facing DGAT), three approaches were used. (1) Dose–response curves were obtained for the effect of increasing taurocholate concentrations on the latency of the activities of DGAT, mannose-6-phosphatase and acyl-CoA: ethanol acyltransferase on the same preparations of microsomes. Figure 2 shows that the half-maximally effective concentration of taurocholate (0.3 %) was identical for the exposure of all three enzymes studied, although, as expected, the increase in enzyme activity upon taurocholate treatment was manyfold higher for the marker enzymes. The similarity between the taurocholate-concentration-dependence of the exposure of the three activities indicates that the increased DGAT activity in the presence of taurocholate results from the physical disruption of the microsomal membrane. (2) Membrane disruption was also performed using alamethicin, which inserts into membranes and produces voltage-dependent pores through the formation of multi-helix aggregates [28] and, in contrast

Table 1 Activities of DGAT and ethanol acyltransferase in microsomal membranes isolated from livers of 24-h-starved rats, before and after permeabilization with alamethicin

DGAT activity was assayed with liposome-delivered dipalmitoyl glycerol (see the Materials and methods section). A low concentration of ethanol (0.7% v/v, final concentration) was present in all incubations to allow assay of transferase activity. Activities are expressed as palmitoyl moieties incorporated into TAG or ethylpalmitate (mean $\pm$ S.E.M.).

	Enzyme activity (nmol/min per mg of protein)		
	Intact	Permeabilized	Ratio (total/overt)
DGAT ($n = 6$)	0.21 $\pm$ 0.05	0.63 $\pm$ 0.10	3.38 $\pm$ 0.60
Ethanol acyltransferase ($n = 6$)	0.13 $\pm$ 0.01	1.30 $\pm$ 0.14	8.64 $\pm$ 1.65



Scheme 1 Metabolic scheme illustrating the role suggested for the overt (I) and latent (II) forms of DGAT in the synthesis and secretion of TAG by rat liver

DAG newly-synthesized through the phosphatidate pathway is proposed to have two fates. It can permeate the ER membrane and be converted into TAG by DGAT II on the lumenal side of the membrane, where it can be incorporated, through the action of microsomal transfer protein during co-translational insertion of apoB, into nascent particles. Alternatively, it is converted into TAG on the cytosolic aspect of the ER membrane through the action of overt DGAT I, and subsequently is incorporated into the cytosolic droplet TAG pool. This is mobilized through hydrolysis to DAG and monoacylglycerides. The DAG generated after remodelling through trans-esterfication, permeates the ER membrane where it is re-esterified to TAG on the lumenal side of the membrane through the activity of latent DGAT II. In the SER this process is envisaged to give rise to non-apoB-associated TAG; in the RER it could contribute to nascent particle formation. The acyl moieties for intralumenal DAG esterification are suggested to be transported across the ER membrane through the combined actions of the overt and latent microsomal membrane carnitine acyltransferases and a putative carnitine-acylcarnitine carrier.

with detergents, alamethicin, and other membrane-destabilizing peptides, interact with membrane phospholipids [29]. Permeabilization of microsomal membranes with alamethicin (Table 1) increased DGAT activity (means $\pm$ S.E.M.) by 3.38 $\pm$ 0.60 fold ($n = 6$) for total/overt DGAT activity. (These data were obtained with the use of dipalmitoylglycerol–phosphatidylglycerol liposomes for the delivery of diacylglycerol substrate, but similar values were obtained with both types of substrate delivery, see the Materials and methods section.) This was higher than the ratio obtained with taurocholate (2.1 $\pm$ 0.5, see above). As the total/overt acyl-CoA:ethanol acyltransferase activity ratio was not increased, these data indicate that the use of taurocholate may cause the latency of DGAT to be underestimated, presumably by inhibition of the latent activity. This may explain why previous attempts at determining the latency of DGAT using taurocholate were inconclusive (see the Introduction). (3) Microsomes were treated with a range of proteases before permeabilization, in order to ascertain whether it was possible to inactivate overt DGAT activity preferentially. However, DGAT activity was found to be remarkably resistant to inactivation by proteases, and membrane integrity was compromised (judged by increased overtness of acyl-CoA:ethanol acyltransferase) before any decrease in DGAT activity occurred (results not shown). Consequently, it was concluded that the topology of DGAT cannot be determined unequivocally by limited-proteolysis experiments [14].

Conclusions

We conclude from these results that separate DGAT activities (designated DGAT I and II respectively) exist on either side of the ER membrane in rat liver. The data obtained with alamethicin suggest that DGAT activity could be up to twice as high on the luminal side of the ER membrane than on the cytosolic side. By contrast, and in agreement with [13–15], the activity of glycerol 3-phosphate acyltransferase was not increased by the presence of 0.3 % taurocholate (Figure 2d), indicating that this enzyme is exclusively overt. Our observations that DGAT has overt and lumenal ER activities suggest the model for TAG synthesis and secretion shown in Scheme 1. The model proposes dual pathways for TAG synthesis and incorporation into VLDL in which overt and latent DGAT activities would play distinct roles. By contrast with the suggestion made in [30], the model depicted in Scheme 1 does not require that all the triacylglycerol-synthesizing enzymes should be duplicated on the cytosolic and lumenal aspects of the ER membrane; only DGAT needs to be present on both sides of the membrane. In this respect, the extensive experiments of Bell and colleagues [13–15] indicated that all the glycerolipid synthesizing enzymes are overt, and we have confirmed this for glycerol 3-phosphate acyltransferase under our conditions (Figure 1). These same authors [15] also made the observation that DGAT was only partially overt (60 % of total activity), although they do not appear to have appreciated its significance and subsequently promulgated the currently held consensus (see e.g. [11]) that TAG synthesis is exclusively overt.

Esterification of DAG on the luminal aspect of the ER membrane necessitates the transfer of both DAG and of acyl moieties across the membrane (Scheme 1). The ability of DAG to permeate biological membranes is well established [31] but acyl-CoA esters are impermeable through lipid bilayers. This was recognized by Yang et al. [11] as a potential obstacle to the type of model shown in Scheme 1 and led those authors to suggest that DAG re-esterification has to occur on the cytosolic face of the membrane (see the Introduction), which would necessitate the transmembrane transfer of TAG. In the RER this may be mediated concomitantly with the co-translational insertion of apoB through the membrane by the action of microsomal transfer protein [32]. However, permeation of the lipid bilayer by TAG independently of apoB is unlikely. The existence of latent DGAT II makes it possible for TAG to be synthesized on the luminal aspect from DAG that permeates the membrane. Conversely, the main function of overt DGAT is suggested to be the provision of TAG destined for cytosolic droplet deposition. The existence of multiple sites of synthesis of TAG and of different pools of both TAG and DAG within the microsomal compartment has been established for some time from kinetic studies on the metabolism

of fatty acids *in vivo* and in isolated liver preparations ([33–36]; reviewed in [3]) and the role of a hydrolysis and re-esterification cycle between tri-, di- and mono-acylglycerols in the generation of secretory TAG has been emphasized by the work of Gibbons and co-workers [1,8–10] and Yang et al. [11,12]. The discovery of cytosol- and lumen-facing forms of DGAT provides an explanation for those findings. Differential regulation of the activities of the two DGATs represents an added tier of control of hepatic TAG secretion at the level of DAG partitioning, between DAG utilization for cytosolic TAG synthesis (including substrate cycling) and sequestration into the ER lumen for synthesis of TAG pools involved in the two-step lipidation of VLDL particles.

With respect to the source of intraluminal acyl-CoA for DGAT II activity, the recent description of overt and latent forms of carnitine acyltransferases in the microsomal membrane [16,17,30] suggests that carnitine-mediated transfer of acyl chains across the membrane could occur (assuming, by analogy with the plasma membrane and mitochondrial inner membrane, that carrier-mediated acylcarnitine transport across the ER membrane occurs). Parallel delivery of acyl moieties and of DAG across the ER membrane would provide both substrates for DGAT II activity.

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