Human acyl-CoA:diacylglycerol acyltransferase is a tetrameric protein

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Acyl-CoA:diacylglycerol acyltransferase (DGAT) is an integral membrane enzyme that catalyses the last step of triacylglycerol synthesis from diacylglycerol and acyl-CoA. Here we provide experimental evidence that DGAT is a homotetramer. Although the predicted molecular mass of human DGAT protein is 55 kDa, CHAPS-solubilized recombinant human DGAT was eluted in fractions over 150 kDa on gel-filtration chromatography. Cross-linking of recombinant DGAT in membranes with disuccinimidyl suberate yielded bands corresponding to the dimer (108 kDa) and the tetramer (214 kDa) in SDS/PAGE. Finally, when two differently epitope-tagged forms of DGAT were co-transfected into mammalian cells, they could be co-immunoprecipitated. From a human adipose tissue cDNA library we cloned a cDNA encoding a novel splice variant of DGAT (designated DGATsv)

that contained a 77 nt insert of unspliced intron with an in-frame stop codon. This resulted in a truncated form of DGAT that terminated at Arg-387, deleting 101 residues from the C-terminus containing the putative active site. DGATsv was enzymically inactive when transfected in HEK-293E cells but was still able to form dimer and tetramer on cross-linking, indicating that the ability to form tetramers resides in the N-terminal region. When co-expressed in HEK-293E cells, DGATsv did not inhibit the activity of full-length DGAT, suggesting that the subunits of DGAT catalyse triacylglycerol synthesis independently.

Key words: differential splicing, membrane protein, protein complex, triacylglycerol.

INTRODUCTION

Acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) is a membrane-bound enzyme that catalyses triacylglycerol formation by the acylation of diacylglycerol. DGAT belongs to the acyl-CoA:cholesterol acyltransferase (ACAT) gene family, which is composed of three membrane-bound acyltransferases: ACAT1, ACAT2 and DGAT [1,2]. The discovery of this gene family was pioneered by Chang and colleagues, who cloned the cDNA of ACAT1 8 years ago [3]. DGAT cDNA was identified subsequently from expressed sequence tag databases through its homology to ACAT1 [4,5]. When mouse DGAT cDNA was expressed heterologously in insect cells, elevated levels of DGAT activity were found in the insect-cell membranes [4].

Triacylglycerol molecules are the major energy-storage form for animals [6,7]. Two biological pathways involving acyl-CoA have been found for triacylglycerol synthesis [8]. In the glycerol phosphate pathway, which is present in all tissues, acyl-CoA is esterified to glycerol 3-phosphate in two consecutive steps to form lysophosphatidic acid and then phosphatidic acid. Phosphatidic acid is hydrolysed to form diacylglycerol, which in turn is acylated by DGAT to form triacylglycerol [9]. The second, monoacylglycerol, pathway is found primarily in enterocytes of the small intestine. In this pathway monoacylglycerol, a major lipolysis product of dietary fat by pancreatic lipase, undergoes two sequential acylation steps by monoacylglycerol acyltransferase and by DGAT to form triacylglycerol [8].

In that the biochemical reaction catalysed by DGAT is the final and only step for triacylglycerol synthesis in both of these two pathways, DGAT had been believed to have an indispensable physiological function in the differentiation of adipose tissue, lipoprotein assembly in liver, and dietary fat absorption in the intestine. This view was challenged when mice deficient in the

gene encoding DGAT [*Dgat*^(-/-)] were created by Farese and co-workers [10]. *Dgat*^(-/-) mice seem to have a fully differentiated fat mass and normal serum triacylglycerol levels, indicating that lipoprotein assembly is normal. They do not seem to have malabsorption for fat because faecal output and fetal caloric content were similar to those in the wild type. These findings illustrate that there is one or more alternative pathways for the synthesis of triacylglycerol [10].

The interesting phenotype exhibited by *Dgat*^(-/-) mice was their resistance to obesity when they were fed with a high-fat diet. Associated with that, they also showed higher sensitivity to insulin. These results raise a possibility that selective inhibition of DGAT might offer a new strategy for treating obesity and obesity-associated insulin resistance [10].

Despite the emerging insights of DGAT function in energy homeostasis in the animal, at the biochemical level, much remains to be explored about DGAT. It has never been purified and its membrane topology and regulation are unknown.

Here we describe the further characterization of DGAT. First, we expressed human DGAT in insect cells and found that DGAT is a homotetramer. Secondly, we identified a novel DGAT transcript encoding a C-terminal deletion of DGAT that can still form tetramers but is not active alone or as a dominant-negative protein.

EXPERIMENTAL

Materials

We obtained monoclonal anti-Myc antibody from Invitrogen; Penta-His antibody from Qiagen; anti-FLAG[®] M2 antibody, anti-FLAG[®] M2 agarose and FLAG peptide (DYKDDDK;

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; CMV, cytomegalovirus; DGAT, acyl-CoA:diacylglycerol acyltransferase; DSS, disuccinimidyl suberate; MOI, multiplicity of infection; PNS, postnuclear supernatant.

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single-letter amino acid codes) from Sigma; goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated antibodies from Pierce; CHAPS from Calbiochem; Complete® protease inhibitor cocktail tablets and Eugene 6 transfection reagent from Roche Molecular Biochemicals; disuccinimidyl suberate (DSS) from Pierce; prepacked Superdex 200 HR 10/30 columns from Amersham Pharmacia Biotech; gel-filtration standards from Bio-Rad; oligonucleotide primers from Sigma-Genosys; human fat-cell 5'-stretch plus cDNA library from Clontech; restriction enzymes from New England Biolabs; LA-PCR kit and ligation kit from Panvera Corporation; QuikChange™ site-directed mutagenesis kit from Stratagene; Bac-to-Bac® baculovirus expression system from Gibco BRL; oleoyl coenzyme A, triolein and 1,2-dioleoyl-*sn*-glycerol from Sigma; [³H]triolein from NEN Life Science Products; [1-¹⁴C]oleoyl-CoA from Amersham Pharmacia Biotech; human embryonic kidney 293-EBNA (Epstein-Barr virus nuclear antigen) (designated HEK-293E) cells and Sf+ cells from DuPont Pharmaceuticals master cell bank.

Construction of plasmids

pCMV-FLAG-human DGAT encodes a 496-residue fusion protein that consists of an initiation methionine residue, a new residue Gly, one copy of the FLAG epitope and human DGAT residues 2–488. Expression is driven by cytomegalovirus (CMV) promoter/enhancer. pCMV-FLAG-human DGAT was constructed as follows. First, an approx. 1.5 kb fragment was amplified from human fat-cell 5'-stretch plus cDNA library by PCR with the following primers: 5' primer, 5'-GGCTGAGG-CCGAATTCGCCACCATGGGCGATTATAAAGATGACGATGACGGCGACCGCGGCAGCTCCCGGCGCCGAGG-3'; 3' primer, 5'-GCCCTCAGGTGCAGCTCTAGATCAGGCCCTGCGCGCTGGGGCCTCATAGTTG-3' (these primers were designed on the basis of sequence information in [5]). Secondly, this 1.5 kb fragment was digested with *Eco*RI and *Xba*I and ligated with an approx. 5.5 kb fragment released from the digestion of pcDNA3 (Invitrogen) with *Eco*RI and *Xba*I. Twelve individual clones of the ligation transformants were picked at random and subjected to sequence analysis. One of them contained a full-length human DGAT open reading frame encoding a protein sequence identical with that reported by Oelkers et al. [5]. This plasmid was designated pCMV-FLAG-human-DGAT.

pCMV-FLAG-human-DGATsv (in which sv stands for splice variant) contained exactly the same nucleotide sequence as pCMV-FLAG-human-DGAT except for an insert of a 77 bp novel nucleotide sequence (GTAGGTGGGGTGTGTGTGTGTGTGTGATGTGGAACATGGCTGTGAACCTGAACCGCTTCCATGCCCCCTCCTCTGCAG) after nt 1160 (counting from the initiation of the open reading frame). pCMV-FLAG-human-DGATsv was another individual ligation transformant that was obtained together with pCMV-FLAG-human-DGAT.

pCMV-FLAG-human-DGATsv (G → A) was identical to pCMV-human-DGATsv, except that G¹¹⁶¹ was replaced with A. To generate pCMV-FLAG-human-DGATsv (G → A) with QuikChange™ site-directed mutagenesis kit, oligonucleotides 5'-CACAAGTGGTGCATCAGATAGGTGGGGTGTGTG-3' and 5'-CACACACCCACCTATCTGATGCACCACTTGTG-3' were used.

pCMV-Myc-human-DGAT encodes a 498-residue fusion protein consisting of an initiation methionine residue, one copy of the 9E10 Myc-epitope derived from human c-Myc (EQKLISEEDL) and human DGAT residues 2–488. The construction of pCMV-Myc-human-DGAT was as follows. First, an approx.

1.1 kb fragment was amplified by PCR from the pCMV-FLAG-human-DGAT template with the following primers: 5' primer, 5'-GGCTGAGGCCGAATTCGCCACCATGGAACAAAACTCATCTCAGAAGAGGATCTGGGCGACCGCGGCA-GCTCCCGGCGCCGAGG-3'; 3' primer, 5'-TGAGGTGACAGACTCGGAG-3'. Secondly, this 1.1 kb fragment was digested with *Eco*RI and *Bsp*EI and the resultant approx. 800 bp fragment was ligated with an approx. 6.2 kb fragment released from the digestion of pCMV-FLAG-human-DGAT with *Eco*RI and *Bsp*EI. Sequence analysis was conducted to confirm the ligation junction and the PCR-amplified sequence.

pBac-FLAG-human-DGAT encoded identical protein to pCMV-FLAG-human-DGAT. pBacHis-FLAG-human-DGAT encoded a 527-residue fusion protein consisting of an initiation methionine residue, three novel amino acids (SYV), six consecutive histidine residues, seven new amino acids (DYDIPTT), one copy of the TEV protease site (ENLYFQG), nine new amino acids (AMDPEFATMG) encoded by a multicloning site sequence, one copy of the FLAG epitope, and human DGAT residues 2–488. pBac-FLAG-human-DGAT and pBacHis-FLAG-human-DGAT were constructed by ligating three pieces of DNA fragment as follows: (1) an approx. 4.7 kb fragment released from the digestion of pFastBac1 (for pBac-FLAG-human-DGAT) and pFastBacHTa (for pBacHis-FLAG-human-DGAT) with *Eco*RI and *Hind*III; (2) an approx. 1.2 kb fragment released from the digestion of pCMV-FLAG-human-DGAT with *Eco*RI and *Apa*LI; (3) an approx. 300 bp fragment released from the digestion with *Apa*LI and *Hind*III of a PCR-amplified product obtained from the pCMV-FLAG-human-DGAT template by using the following primers: 5' primer, 5'-GGCAGAACTGGAACATCCCTGTGC-3'; 3' primer, 5'-TCTAGAAAGCTTTCAGGCCTCTGCCGCTGG-3'. The ligation junctions and PCR-amplified sequence were confirmed by sequence analysis.

Transfection of HEK-293E cells

The transfection method with Eugene reagent was adapted from Rawson et al. [11], with slight modifications. Monolayer cultures of HEK-293E cells were set up for experiments on day 0 (5×10^5 cells per 25 cm² flask) and cultured in air/CO₂ (19:1) at 37 °C in medium A [DMEM containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 400 µg/ml G418, 100 i.u./ml penicillin and 100 µg/ml streptomycin sulphate]. On day 2, cells were refed with 4 ml of medium A. For each transfection the indicated amount of plasmid and Eugene reagent mixed at a ratio of 3 µl of Eugene to 1 µg of DNA were incubated with 0.2 ml of prewarmed DMEM (without antibiotics) for 30 min. The mixture was then added to each flask. On day 4 the transfected cells were harvested for further analysis.

Expression of human DGAT with insect-cell baculovirus system

To construct strains of recombinant baculoviruses that express FLAG-human-DGAT and His-FLAG-human-DGAT, pBac-FLAG-human-DGAT and pBacHis-FLAG-human-DGAT were used as transfer vectors respectively by following the manufacturer's instructions for Bac-to-Bac® baculovirus expression systems. To express recombinant human DGAT protein, Sf+ cells were infected at a multiplicity of infection (MOI) of three plaque-forming units per cell in medium B [Sf-900II serum-free medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin sulphate]. At 48 h after infection, the cells were harvested and the 10⁵ g subcellular membrane fraction was

isolated (by centrifugation at $10^5 g$ for 1 h at 4 °C) for immunoblot analysis or for the assay of DGAT *in vitro*.

Antibodies and immunoblot analyses

For immunoblot analyses, 5 µg/ml anti-FLAG® M2, 2 µg/ml anti-Myc and 0.2 µg/ml Penta-His antibody were used. Bound antibodies were revealed with horseradish peroxidase-conjugated affinity-purified secondary antibodies by using the SuperSignal CL-HRP substrate (Pierce) in accordance with the manufacturer's instructions. Blots were exposed to Kodak BioMax film at 24 °C.

Immunoprecipitation of FLAG-epitope-tagged DGAT

On day 0 HEK-293E cells were seeded at 5×10^5 cells per 25 cm² flask in medium A. On day 2 the cells were transfected with DGAT expression vectors with Fugene reagent as described above. On day 4 the transfected cells were harvested and the $10^5 g$ subcellular membrane fraction was isolated. The membrane pellet pooled from three flasks of transfected cells was then resuspended in 1 ml of buffer A [50 mM Tris/HCl (pH 8.5)/150 mM NaCl/1% (v/v) CHAPS/5 µg/ml pepstatin A/10 µg/ml leupeptin/0.5 mM Pefabloc/3 µg/ml aprotinin/5 mM EDTA/5 mM EGTA/2 mM benzamidin] by passing it 15 times through a 23-gauge needle. This membrane resuspension was recentrifuged at $10^5 g$ for 1 h at 4 °C. An aliquot of the supernatant (1/10 of the total) was saved for SDS/PAGE and immunoblot analyses as immunoprecipitate input. The remaining supernatant were mixed with 30 µl of FLAG M2 agarose beads and rocked gently for 2 h. The immunoprecipitate pellets were washed seven times with 1 ml of buffer A. The proteins bound to the beads were then eluted with 100 µl of buffer A supplemented with 100 µg/ml FLAG peptide for 30 min by gentle rocking. Aliquots of the eluate were subjected to SDS/PAGE and immunoblot analysis for the immunoprecipitate pellet. The entire procedure of immunoprecipitation was performed at 4 °C.

Assay of DGAT *in vitro*

The DGAT assay was performed as described [12] with slight modification. Either HEK-293E cells or Sf+ cells were homogenized by sonication with a probe sonicator in buffer B [5 mM Tris/HCl (pH 7.5)/250 mM sucrose/Complete® protease inhibitor cocktail tablet] at 4 °C. The cell homogenates were centrifuged at 1000 g for 10 min. For HEK-293E cells the postnuclear supernatant (PNS) was used for the DGAT assay. For Sf+ cells the PNS was centrifuged at $10^5 g$ for 30 min and the membrane pellet was resuspended in assay buffer [175 mM Tris/HCl (pH 7.8)/0.2 mg/ml BSA/8 mM MgCl₂] for the DGAT assay.

To perform DGAT assays, 5–20 µg of protein from the PNS or the membrane pellet was preincubated with 200 µl of assay buffer supplemented with 160 µM 1,2-dioleoylglycerol [in acetone, final vehicle concentration 5% (v/v)] for 10 min in a water bath at 23 °C. The DGAT reaction was initiated by adding 10 nmol of [¹⁴C]oleoyl-CoA (specific radioactivity 10000 d.p.m./nmol, stock concentration 1 nmol/µl). After incubation for 10 min at 23 °C, the reaction was terminated by the addition of 6 ml of chloroform/methanol (2:1, v/v). An internal standard with carrier ([³H]triolein, 2000 d.p.m./µl, 1 µg/µl in toluene) was added to determine the recovery of triacylglycerol in the subsequent steps. To facilitate phase separation, 1.2 ml of water was added, mixed and left at room temperature for more than 2 h. The aqueous phase was discarded and the organic phase containing the lipids was dried under N₂, resuspended in 100 µl of chloroform and spotted on ITLC-SA thin-layer plates (Gelman

Sciences). Lipids were separated by TLC with a solvent system of hexane/diethyl ether/acetic acid (85:15:0.5, by vol.) for 20 min. The lipids were revealed by staining with iodine; the triacylglycerol band was cut from the plate and subjected to scintillation counting. Specific DGAT activity was calculated as nmol/min per mg of protein.

Gel-filtration chromatography

Sf+ cells were infected with recombinant FLAG-DGAT baculovirus; the $10^5 g$ membrane fraction was isolated as described above. An aliquot of membrane fraction (from 5×10^6 cells) was resuspended with 1 ml of buffer A by passing it 15 times through a 23-gauge needle. This CHAPS membrane resuspension was centrifuged at $10^5 g$ for 1 h at 4 °C; 200 µl of the supernatant was injected into a buffer-A-equilibrated Superdex 200 HR 10/30 gel-filtration column (Amersham Pharmacia Biotech). The chromatography was conducted on an FPLC system (Amersham Pharmacia Biotech) at a flow rate of 0.25 ml/min in buffer A. Fractions of 0.5 ml were collected. Molecular masses were calibrated in the same buffer with Gel Filtration Standard (Bio-Rad) consisting of thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and cyanocobalamin (1.4 kDa). All procedures of chromatography were performed at 4 °C.

Cross-linking of DGAT in the membrane

Sf+ cells or HEK-293E cells were infected or transfected to express DGAT as indicated above. The cells were harvested and washed with PBS, and then homogenized in ice-cold buffer C [10 mM Hepes (pH 7.4)/5 mM EDTA supplemented with Complete® protease inhibitor cocktail tablet] with a probe sonicator. The $10^5 g$ membrane pellets were resuspended with buffer C by passing them 15 times through a 23-gauge needle. Various quantities of DSS [in DMSO, vehicle concentration 3% (v/v)] were then added to the membrane resuspension and incubated for 20 min at room temperature. The reactions were quenched by adding 1/10 vol. of 1 M Tris/HCl, pH 8.0. Aliquots of the reactions were subjected to SDS/PAGE and immunoblotted with anti-FLAG M2 IgG. The molecular masses of cross-linking products were calculated by reference to molecular mass markers (SeeBlue® prestained standard; Invitrogen) by using Cricket software.

RESULTS

We used Sf+ cells to produce two versions of recombinant DGAT proteins, one with an N-terminal FLAG tag and the other with a His-FLAG tag (Figure 1A). In contrast with wild-type baculovirus (Figure 1B, lane 1), FLAG-DGAT and His-FLAG-DGAT produced specific bands consistent with predicted molecular masses (Figure 1B, lanes 2 and 3). The His tag caused an increase in size of the His-FLAG-DGAT fusion protein compared with FLAG-DGAT when it was expressed in the insect cells, as confirmed by an anti-His immunoblot (Figure 1C, lane 6).

To examine whether these recombinant DGAT proteins expressed in insect cells were functionally active, the membrane fractions were used to perform DGAT assays *in vitro*. Figure 1(D) shows that membranes from Sf+ cells infected with recombinant DGAT viruses produced DGAT activities approx. 10-fold those from cells infected with wild-type virus or from non-infected cells. Inclusion of the His tag did not make a great difference to the specific activity of DGAT.

Figure 1 Expression of human DGAT in Sf+ cells by recombinant baculovirus

(A) Schematic diagram of human DGAT fusion proteins expressed in baculoviruses shown by the horizontal bars; the numbers correspond to the amino acid residues deduced from human DGAT cDNA. The strains of the corresponding recombinant viruses are designated FLAG-DGAT and His-FLAG-DGAT respectively. '6 × His' denotes six consecutive histidine residues. (B, C) Immunoblot analyses of membrane fractions of Sf+ cells infected with wild-type, recombinant FLAG-DGAT and His-FLAG-DGAT baculoviruses. On day 0 Sf+ cells were set up for experiments at a density of 5×10^6 cells per 25 cm² flask in medium B. On day 1 the cells were infected with wild-type and recombinant viruses at a MOI of 3. On day 3 the infected cells were harvested and membrane fractions were isolated as described in the Experimental section. Aliquots of the 10^5 g membrane fraction (5 µg of protein) were subjected to SDS/PAGE and immunoblotted with 5 µg/ml anti-FLAG[®] M2 monoclonal IgG (B) or 0.2 µg/ml of Penta-His IgG (C). The filters were exposed for 1 s. The positions of molecular mass standards are indicated at the left. (D) DGAT *in vitro* assay of Sf+ cells with or without infection with the baculoviruses. Sf+ cells were infected and membrane fractions were isolated as described in (B). Aliquots of the membrane fraction (10 µg of protein) were subjected to DGAT assay *in vitro* as described in the Experimental section. Specific activities of DGAT are expressed as nmol of triacylglycerol formed/min per mg of protein. Duplicate assays were performed; variation was within 10%.

To determine whether DGAT is a multimeric protein, we estimated its molecular mass by gel filtration. As shown in Figure 2(A) we solubilized the membrane fraction isolated from FLAG-DGAT-infected Sf+ cells with a buffer containing 1% CHAPS. CHAPS is known to solubilize native DGAT without disrupting its enzymic activity [13]. Aggregates were removed by centrifugation at 10^5 g for 1 h and the supernatant was subjected to chromatography on a Superdex 200 gel-filtration column. Fractions were collected and immunoblotted. Figure 2(A) shows that DGAT was eluted at a volume of 9–11 ml, corresponding to a broad spectrum of molecular masses ranging from 150 to 670 kDa. In fractions corresponding to lower molecular masses no DGAT signal was detected.

The broad peak of the elution profile suggests that DGAT enzyme is heterogeneous in molecular mass in detergent solution. It is likely that DGAT is associated with detergent micelles and lipids. To address whether protein–protein interaction might explain why DGAT was eluted in high-molecular-mass fractions, we performed a co-immunoprecipitation experiment. In Figure 2(B), FLAG-tagged and Myc-tagged DGAT were transiently expressed either alone or together in HEK-293E cells. Anti-FLAG-agarose beads were used to immunoprecipitate FLAG-tagged DGAT from the CHAPS-solubilized membrane fraction. When Myc-DGAT was expressed alone (lane 3), no Myc-DGAT was detected by immunoprecipitation because anti-FLAG-agarose beads do not cross-react with Myc-DGAT. In contrast, when Myc-DGAT was co-expressed with FLAG-DGAT, a large amount of Myc-DGAT was immunoprecipitated (lane 4). Myc-DGAT therefore forms a complex with FLAG-DGAT.

Figure 2 DGAT is a complex when solubilized with CHAPS

(A) Gel-filtration chromatography of DGAT protein solubilized with CHAPS. Sf+ cells were infected with recombinant FLAG-DGAT baculovirus and the membrane fraction was isolated as described in the legend to Figure 1. An aliquot of the membrane fraction (from 5×10^6 cells) was resuspended in 1 ml of buffer A. This lysate was centrifuged at 10^5 g for 1 h at 4 °C; 200 µl of the resultant supernatant was subjected to Superdex 200 gel-filtration chromatography as described in the Experimental section. Fractions of 0.5 ml were collected and aliquots (30 µl per fraction) of them were subjected to SDS/PAGE and immunoblotted with anti-FLAG[®] IgG. The filter was exposed for 2 s. The positions of molecular mass standards are indicated at the top. V₀, void volume. (B) Immunoprecipitation of the DGAT complex. On day 0 HEK-293E cells were seeded at 5×10^5 cells per 25 cm² flask. On day 2 each flask of cells was transfected with 1 µg of pCMV-FLAG-human-DGAT (lane 2), 1 µg of pCMV-Myc-human-DGAT (lane 3) or 1 µg of pCMV-FLAG-human-DGAT and pCMV-Myc-human-DGAT (lane 4) as described in the Experimental section. Sufficient empty vector pcDNA3 was added to equalize the total transfected DNA at 2 µg per flask. On day 4 the transfected cells were harvested and immunoprecipitation (IP) was performed with FLAG M2[®] agarose beads as described in the Experimental section. Aliquots of 30 µl of CHAPS membrane lysate (for IP input) and 30 µl of immunoprecipitation elution (for IP pellet) were subjected to SDS/PAGE and immunoblotted with anti-FLAG and anti-Myc IgG. The blots were exposed for 3 s for IP input and 1 s for IP pellet respectively.

The results in Figure 2 are consistent with the hypothesis that DGAT is multimeric but they do not address how many copies of DGAT the complex contains, owing to the inaccuracy of estimating membrane protein size in detergent by gel filtration. To circumvent this limitation, we established conditions for cross-linking membrane-bound DGAT. As shown in Figure 3, the membrane fraction of Sf+ cells infected with FLAG-DGAT was first isolated in an amine-free buffer. The membrane was then exposed to various concentrations of DSS, a non-cleavable, amine-reactive, homobifunctional cross-linker with a spacer arm of 11.4 Å. The cross-linking reaction was then quenched with a high concentration of Tris base and the mixture was immunoblotted with anti-FLAG IgG. The molecular masses of the immunoreactive bands in Figure 3(A) were calculated relative to standards (Figure 3B). In Figure 3(A), in addition to the predicted monomeric DGAT at 53 kDa, two more immunoreactive bands at molecular masses of 108 and 214 kDa appeared when the DSS concentration exceeded 10 nM (lane 5). We conclude that these two additional bands were cross-linked products of DGAT corresponding to the homodimer (108 kDa) and homotetramer (214 kDa) respectively.

Figure 3 Cross-linking of membrane-bound DGAT

(A) Dose-dependent cross-linking of recombinant DGAT with DSS. On day 0, Sf+ cells were seeded and infected for 48 h with baculoviruses at a MOI of 0.3. The 10^5 g membranes were prepared and cross-linking reactions were performed as described in the Experimental section. Aliquots of the reactions (5×10^4 cells per lane) were subjected to SDS/PAGE and immunoblotted with 5 μ g/ml anti-FLAG[®] M2 IgG. The filter was exposed for 3 s. The positions of molecular mass standards are indicated at the left. (B) Calibration of molecular masses of DGAT cross-linking products. Distances of migration for molecular mass markers (SeeBlue[®] prestained standard; Invitrogen) were measured from the origin. They were 1.35, 2.55, 3.15, 3.60 and 4.15 cm for molecular masses of 250 kDa (myosin), 98 kDa (BSA), 64 kDa (glutamate dehydrogenase), 50 kDa (alcohol dehydrogenase) and 36 kDa (carbonic anhydrase) respectively. The equation of distance (D) as a function of molecular mass (M) was derived by Cricket software as $D = 607.056 \times 10^{-0.302M}$ ($r^2 = 0.994$). With this equation, the molecular masses of DGAT cross-linking products with distances of migration of 3.50, 2.35 and 1.50 cm were determined as 53, 118 and 214 kDa respectively. The graph shows a semilogarithmic plot of molecular mass (kDa) against distance (cm). Symbols: ●, molecular mass standards; ○, DGAT cross-linking products, designated 1x, 2x and 4x respectively.

During the isolation of human DGAT cDNA, we found two clones with a 77 bp stretch of new nucleotide sequences inserted after nt 1160. For convenience of discussion we designate the DGAT cDNA that is identical to the published sequence as Transcript1, and the newly identified cDNA as Transcript2. Figure 4(A) shows the nucleotide and amino acid sequences for Transcript1 and Transcript2 in the region where the discrepancy occurs. The new stretch of 77 bp nucleotides in Transcript2 starts with GT and ends with AG, the classic intron boundary sequences that serve as splicing donor and acceptor sites. The insertion of this sequence also predicts a truncation of DGAT at residue 387 by an in-frame stop codon. To verify the production of a truncated protein, we cloned Transcript2 into an expression vector and transfected it into HEK-293E cells. As predicted, we observed a C-terminally truncated protein that was detectable by antibodies against the N-terminal FLAG tag. In addition, we detected a protein that migrated with the same molecular mass as wild-type DGAT encoded by Transcript1 (Figure 4B, compare lanes 2 and 3). We surmised that this could have been due to partial splicing of the 77 nt intron. To prove this, we mutated the splice donor site GT to AT (see Figure 4A) and expressed the resulting construct. As shown in Figure 4(B), lane 4, this mutation prevented splicing and resulted in the expression of the truncated DGAT only.

In the rest of this paper, truncated DGAT is referred to as DGATsv. To establish whether DGATsv still possessed DGAT activity, we transfected HEK-293E cells with expression vectors for DGAT and DGATsv. To achieve a similar level of expression to that of DGAT, approx. 3-fold more plasmid needed to be transfected for DGATsv, suggesting that DGATsv might be less

stable than DGAT. To expedite the procedure from harvesting cells to the DGAT assay *in vitro*, we isolated the PNS from the transfected cells and used it directly to assay DGAT activity. Naive HEK-293E cells and mock-transfected cells exhibited very low DGAT activity (0.2 nmol/min per mg). For cells transfected with DGAT, the amount of immunostained full-length DGAT increased along with the amount of transfected plasmid (Figure 5A, samples 3–6). In parallel, the PNS isolated from the same cells exhibited increased DGAT activity (from 3.5 to 13.4 nmol/min per mg), significantly elevated over background. In contrast, cells transfected with DGATsv expressed immunostainable DGATsv but with no associated DGAT activity. We conclude from this experiment that DGATsv did not possess DGAT activity.

We next addressed whether the C-terminus of DGAT was required for DGAT to form a tetramer. Figure 6(A) shows a cross-linking experiment similar to that in Figure 3. When the membrane fraction of HEK-293E cells expressing DGATsv was exposed to DSS at 0.1 and 1 μ M (Figure 6A, lanes 11 and 12), as with full-length DGAT (lanes 7 and 8), DGATsv was cross-linked into other two species corresponding to its dimeric and tetrameric forms respectively. We conclude that the N-terminus of DGAT was responsible for DGAT tetramer formation and the C-terminus was dispensable.

Because DGATsv formed a homodimer and homotetramer by itself, it was logical to speculate that DGAT and DGATsv might form a heterocomplex when expressed together. To detect a DGAT–DGATsv heterocomplex, we performed a cross-linking experiment similar to that in Figure 6(A), in which we co-expressed DGAT and DGATsv. However, because DGAT and

Figure 4 Identification of a cDNA for DGATsv

(A) Amino acid and nucleotide sequences of human DGAT in the region in which a 77 nt stretch was inserted. The cDNA identical with the published sequence is designated Transcript1; the cDNA with the insert is designated Transcript2. The mutated cDNA of Transcript2 bearing a transition of the first G in the insert to A is designated Transcript2 (G → A). (B) Expression of DGAT and DGATsv in HEK-293E cells. On day 0 HEK-293E cells were seeded at a density of 5×10^5 cells per 25 cm² flask. On day 2 each flask of cells was transfected with 0.3 μ g of pCMV-FLAG-human-DGAT, 2 μ g of pCMV-FLAG-human-DGATsv and 2 μ g of pCMV-FLAG-human-DGATsv(G → A), for Transcript1, Transcript2 and Transcript2 (G → A) respectively. On day 4 the cells were harvested and 10^5 g membrane fractions were isolated as described in the Experimental section. Aliquots of the membrane (20 μ g of protein per lane) were subjected to SDS/PAGE and immunoblotted with anti-FLAG M2 IgG. The blot was exposed for 2 s. The positions of molecular mass standards are indicated at the left.

DGATsv homodimers and homotetramers appeared as broad bands in an SDS/PAGE gel, we failed to detect discrete bands corresponding to the DGAT–DGATsv heterodimer and heterotetramer (results not shown). We then designed a co-immunoprecipitation experiment as an alternative approach. As in Figure 2(B), we co-transfected FLAG-DGATsv and Myc-DGAT into HEK-293E cells (Figure 6B, lane f). When anti-FLAG–agarose beads were used to immunoprecipitate the FLAG-tagged DGATsv, co-expressed full-length Myc-DGAT was co-precipitated. The amount of Myc-DGAT recovered in the immunoprecipitate pellet was similar to that with FLAG-DGAT as the co-expression partner (Figure 6B, lanes e and f). This result indicates that DGATsv was capable of forming a heterocomplex with full-length DGAT.

The results in Figure 6(B) raise the question of whether DGATsv inhibits DGAT enzymic function once it is complexed with full-length DGAT. To test this possibility we first expressed a basal level of DGAT, readily stained in an immunoblot with

Figure 5 DGATsv is enzymically inactive

On day 0 HEK-293E cells were seeded at a density of 5×10^5 cells per 25 cm² flask in medium A. On day 2, each flask of cells was transfected with 0.03, 0.1, 0.3 or 1 μ g of pCMV-FLAG-human-DGAT (samples 3, 4, 5 and 6 respectively) or with 0.3, 1, 2 or 3 μ g of pCMV-FLAG-human-DGATsv(G → A) (samples 7, 8, 9 and 10 respectively) as described in the Experimental section. For sample 1, the cells were refed with 4 ml of fresh medium A without transfection. For samples 2–9, sufficient pcDNA3 was added to equalize the total transfected DNA at 3 μ g per flask. On day 4 pools of three flasks of cells were homogenized with a probe sonicator for 1 min in 300 μ l of ice-cold assay buffer (without BSA). (A) Aliquots of PNS fraction (20 μ g of protein) were subjected to SDS/PAGE and immunoblotted with anti-FLAG M2. The blot was exposed for 3 s. The positions of molecular mass standards are indicated at the left. (B) Aliquots of PNS fraction (20 μ g of protein) were subjected to DGAT assay *in vitro* as described in the Experimental section. Assays were performed in duplicate; variation was within 10%.

anti-FLAG IgG (Figure 7A, sample 2). The transfected DGAT was sufficient to produce DGAT activity (5.4 nmol/min per mg) approx. 10-fold the background (0.53 nmol/min per mg). This result indicates that the DGAT assay *in vitro* measured activity that was primarily derived from transfected DGAT. In samples 3–6 (Figure 7) we co-transfected increased amounts of DGATsv. Although much larger amounts of DGATsv than DGAT were produced, as judged by immunoblot (Figure 7A, samples 5 and 6), they did not alter the DGAT activity (Figure 7B, samples 5 and 6). This result indicates that DGATsv did not act as a dominant-negative protein and suggests that individual subunits within the tetrameric complex of DGAT might have been independently able to perform the enzymic reaction.

DISCUSSION

The current study provides two lines of evidence that DGAT forms a homotetramer: (1) two differently epitope-tagged DGAT were co-immunoprecipitated, and (2) membrane-bound DGAT was cross-linked to dimeric and tetrameric forms with a cross-linking agent.

Highly purified ACAT1 forms a homotetramer [14,15], indicating that no other bridge protein is required for its subunits to interact. The purified ACAT1 also preserves its property of an

A Crosslinking of DGAT and DGATsv in HEK-293E cells **B** Immunoprecipitation of DGAT-DGATsv heterocomplex with Anti-FLAG IgG **Figure 6** DGATsv is able to form a homotetramer and a heterocomplex with full-length DGAT

HEK-293E cells were transfected with the indicated plasmids as described in the legend to Figure 4. (A) Cross-linking experiments were conducted as described in the Experimental section. The positions of molecular mass standards are indicated at the left. (B) Membrane lysates solubilized with CHAPS were used for immunoprecipitation with FLAG-agarose as described in the legend to Figure 2(B).

allosteric enzyme [15]. In mixed micelles or in preformed vesicles, the cholesterol substrate-concentration curve of ACAT1 is highly sigmoidal [15,16]. It was suggested that through protein-protein interaction the binding of cholesterol with one subunit induces its neighbouring subunit to acquire higher affinity for cholesterol. Hence complex formation might be important for the allosteric regulation of ACAT1. However, the biochemical significance of tetramer formation of DGAT is not yet known. It might be possible that DGAT also interacts with its substrate, diacylglycerol, in a co-operative manner. To address this possibility requires the development of a DGAT assay system in a mixed micelle or preformed vesicle that allows the efficient delivery of hydrophobic substrate, as Chang has done for ACAT [16].

The tetrameric structure of DGAT resembles its close homologue ACAT1 [14,17,18]. The fine structural requirements for tetramer formation by either DGAT or ACAT remains to be determined. It has been recognized that a putative leucine-zipper

Figure 7 DGATsv does not inhibit the co-expressed DGAT

HEK-293E cells were set up and transfected and the DGAT assay was conducted *in vitro* as described in the legend to Figure 5. For β -galactosidase expression, pcDNA3.1-His/*lacZ* (Invitrogen) was used. (A) Blots were stained with 0.2 μ g/ml Penta-His IgG (upper panel) and with 5 μ g/ml anti-FLAG[®] M2 IgG (bottom panel) and exposed for 5 and 2 s respectively. (B) DGAT assays were performed in triplicate; variation was within 10%.

motif resides near the N-terminus of ACAT1 [3], suggesting that the N-terminal part might mediate its complex formation. This putative leucine-zipper motif is not preserved in DGAT. However, the N-terminal portion of DGAT does seem to mediate complex formation because DGATsv, with its deletion of the last 101 residues of DGAT, forms a homotetramer and a heterocomplex with full-length DGAT.

We also report a novel DGAT splice variant, designated DGATsv. This cDNA has an insertion of a 77 nt unspliced intron. The insert introduces a stop codon in frame after Arg-387 and truncates the DGAT protein by deleting 101 residues from the C-terminus. This truncation disrupts the latter half of the putative diacylglycerol/phorbol-ester-binding signature sequence that resides at residues 382–392 (motif H.[FWY]..[KR].F..P) [5]. As a result of this truncation, DGATsv does not catalyse the DGAT reaction.

The physiological function of DGATsv remains unknown. Functionally inactive proteins are often reported to possess dominant-negative inhibitory activity by binding to their partners [19,20], but DGATsv did not inhibit DGAT activity when the two forms were co-transfected. DGAT enzymic activity might therefore not require oligomerization of the subunits. In the co-transfection experiment, although expression levels of DGATsv were very high compared with DGAT, we cannot exclude the possibility that a dimeric subunit is important, because we do not have evidence that DGATsv can form 3:1 DGATsv-DGAT complexes. The formation of only heterodimers could still allow homodimeric interactions within the tetramer.

The unspliced intron in DGATsv has 77 nt, which is believed to be relatively short to accommodate spliceosome assembly without steric hindrance. In addition, this intron does not have

any stretch of sequence that absolutely matches the branch-point consensus base sequence YNYURAY [21]. The combination of its shortness and its lack of a strong branch-point might explain why this intron is inefficiently spliced. Splicing of this intron thus creates an additional mechanistic device to regulate the level of expression of functional DGAT between different tissues or under specific physiological conditions. We have found (Meegalla, R. L. and Cheng, D., unpublished work) that the 'intron retention' phenomenon was also observed in various mouse tissues. To demonstrate unambiguously the regulation of splicing of DGAT demands a higher-titre DGAT antibody to detect endogenous DGATsv or a ribonuclease protection assay to measure quantitatively the frequency of occurrence of 'intron retention' in comparison with complete splicing. We are creating these reagents and plan to address these questions.

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