Identification of Proteins That Interact with Acyl CoA:Diacylglycerol Acyltransferase (DGAT) Enzymes

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ABSTRACT

Triacylglycerols are the predominant storage form of energy in eukaryotes. As obesity has become a worldwide problem and excessive accumulation of triacylglycerols in adipose tissue causes obesity, enzymes catalyzing the synthesis of triacylglycerols are of great interest. Acyl CoA:diacylglycerol acyltransferase (DGAT), including the isoforms DGAT1 and DGAT2, catalyze the final and committed step in triacylglycerol synthesis. Proteins that physically interact with DGAT1 may provide information regarding the metabolic role of DGAT1. We chose HEK-293T cell line to express DGAT1 and used mass spectrometry to identify proteins that co-immunoprecipitated with DGAT1. We confirmed that DGAT2 and ACAT1 did interact with DGAT1. The interaction of DGAT1 with DGAT2 appeared to interrupt the synthesis of triacylglycerol since the co-expression of DGAT1 and DGAT2 was expected to increase triacylglycerol synthesis. This implied that DGAT1 and DGAT2 might serve different functional roles. On the other hand, DGAT1 overexpression may increase the synthesis of cholesterol esters that was the product of ACAT1. Additionally, ACAT1 overexpression did increase triacylglycerol synthesis and ACAT1 disruption by siRNA did decrease triacylglycerol synthesis. Our findings indicated that DGAT1 and ACAT1 might be involved in the same lipid-synthesizing protein complex.

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LIST OF ABBREVIATIONS

AAT	Acyl-CoA Acyltransferase
ACAT	Acyl-CoA:Cholesterol Acyltransferase
ARAT	Acyl-CoA:Retinol Acyltransferase
ATP	Adenosine Triphosphate
BY-2	Bright-Yellow 2
cAMP	Cyclic Adenosine Monophosphate
C/EBP	CCAAT/Enhancer-Binding Protein
CE	Cholesterol Ester
CMV	Cytomegalovirus
CoA	Coenzyme A
COS7	African Green Monkey Kidney Cells
DG	Diacylglycerol
DGAT	Acyl-CoA:Diacylglycerol Acyltransferase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
FA	Fatty Acid
FAR	Fatty Acyl-CoA Reductase
G3P	Glycerol-3-phosphate
GPAT	Glycerol Phosphate Acyl-CoA Acyltransferase
HEK-293T	Human Embryonic Kidney 293T Cell Line
HDL	High-density Lipoprotein
IDL	Intermediate-density Lipoprotein
IP	Immunoprecipitation
kDa	Kilodalton
LB	Luria-Bertani

LDL	Low-density Lipoprotein
LPA	Lysophosphatidate
LPAT	Lysophosphatidate Acyltransferase
LRAT	Lecithin:Retinol Acyltransferase
MAM	Mitochondria-associated Membrane
MAPK	Mitogen Activated Protein Kinase
MBOAT	Membrane-bound O-acyltransferase
MG	Monoacylglycerol
MGAT	Acyl-CoA:Monoacylglycerol Acyltransferase
3T3-L1	Mouse Embryonic Fibroblast Cell Line
O.D.	Optical Density
PA	Phosphatidate
PAGE	Polyacrylamide Gel Electrophoresis
PAP	Phosphatidate Phosphatase
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PPAR	Peroxisome Proliferator-activated Receptor
SCD	Stearoyl-CoA Desaturase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
siRNA	Small Interfering RNA
TAE	Tris-Acetate EDTA Buffer
TB	Terrific Broth
TG	Triacylglycerol
TMD	Transmembrane Domain

Tris	Tris-[Hydroxymethyl]-aminomethane
VLDL	Very Low-density Lipoprotein

1. **INTRODUCTION**

Triacylglycerols (TGs) are the major form of stored energy in animals, plant seeds and microorganisms. TGs are composed of a glycerol backbone and three fatty acids (FAs, carboxylic acids with chain containing 4 to 36 carbons) attached by ester bonds (Lehner and Kuksis, 1996). TGs are highly reduced. Oxidation of TGs yields more than twice the free energy relative to an equal mass of carbohydrate. Moreover, TGs are anhydrous and hence lighter, unlike polysaccharides, which need to carry the extra weight of water for hydration (2 g water per gram of polysaccharide). Due to these advantages, almost all tissues have the ability to synthesize TGs, although adipose tissue is the main place for TG storage. Excessive accumulation of TG in adipose tissue leads to obesity and obesity-related disorders, such as diabetes and cardiovascular disease. In order to prevent the excessive accumulation of TG, it is of importance to study the synthesis of TG.

In mammals, there are two different pathways to synthesize TGs, the monoacylglycerol pathway and the glycerol-3-phosphate pathway (Kennedy, 1957; Bell and Coleman, 1980). The committed step for TG synthesis in both pathways is the conversion of diacylglycerol (DG) to TG, catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT). Two different DGAT enzymes have been identified, DGAT1 and DGAT2. Although they catalyze the same reaction, DGAT1 and DGAT2 have distinct physiological functions. DGAT1 and DGAT2 are from different gene families and share no sequence similarity at all. Moreover, DGAT1 possesses multiple enzyme activities, such as acyl-CoA:monoacylglycerol acyltransferase (MGAT), wax synthase and acyl-CoA:retinol acyltransferase (ARAT) activity (Yen *et al*., 2005). In contrast, DGAT2 only has DGAT activity. Physiological studies revealed that DGAT2 is the key enzyme for bulk TG synthesis, as DGAT2 deficiency is lethal to mice (Stone *et al*., 2004). On the contrary, DGAT1 deficient mice are viable (Smith *et al*., 2000).

Through the use of molecular tools, much progress has been made in understanding DGAT biochemical and cellular functions. However, TG synthesis and its incorporation into lipid droplets and secretion into the bloodstream are still poorly characterized cellular events. Since almost every cellular process requires protein-protein interactions, we hypothesize that DGAT1 and DGAT2 interact with other proteins to promote the efficient synthesis of TG. The

objectives of my MSc research studies were to identify DGAT-interacting proteins and examine the roles of them in TG synthesis, storage and/or secretion.

2. LITERATURE REVIEW

2.1 Triacylglycerol

Triacylglycerols (TGs) belong to the class of neutral lipids. A TG molecule is composed of glycerol with all three glycerol hydroxyl groups esterified by a fatty acyl group. Fatty acids consist of a hydrocarbon chain with a terminal carboxyl group. Fatty acids vary in length, shape (straight, branched or ringed) and degree of unsaturation (number of double bonds). In mammals, the fatty acyl chains in the C1 positions of TG are usually straight and saturated. The fatty acyl chain in position 3 of the glycerol backbone is variable, and the most common forms are saturated and monounsaturated fatty acids.

Different species, such as many types of bacteria and yeast, seeds of plants and most animals utilize TG as a major form of energy storage (Alvarez and Steinbuchel, 2002; Sorger and Daum, 2002; Hobbs *et al*., 1999; Cases *et al*., 1998a). There are several advantages for organisms to choose TG over starch or glycogen. First, TGs are highly reduced and reserve more energy compared to sugars or proteins. Completely oxidizing 1 molecule of glucose, glutamine or palmitate (a fatty acid with 16 carbons) produces 38, 24 and 106 molecules of ATP, respectively (Vasta *et al*., 1993). Secondly, TGs are highly hydrophobic that decreases storage mass. On the contrary, every gram of glycogen needs to bind 2 grams of water. Also, TGs are chemically inert and do not react with other molecules.

In humans, TGs are stored in most organs, such as white adipose tissue, liver, muscle and kidney. But TGs are mainly deposited in the white adipose tissue, which is widely distributed under the skin, in the mammary gland and the abdominal cavity. Adipocytes are specialized cells in white adipose tissue responsible for TG synthesis and storage. The characteristic feature of adipocytes is that in the cell, a single large lipid droplet containing TGs takes up most of the intracellular space. TGs in various tissues are obtained from the diet or synthesized, *de novo*. The general absorption of dietary TGs are described in Figure 2.1. After having a fatty meal, bile salts emulsify dietary fats in the small intestine. Pancreatic lipase then hydrolyzes TGs into 2-monoacylglycerol (2-MG) and fatty acids, both of which are taken up by enterocytes and re-esterified by DGAT into TGs.

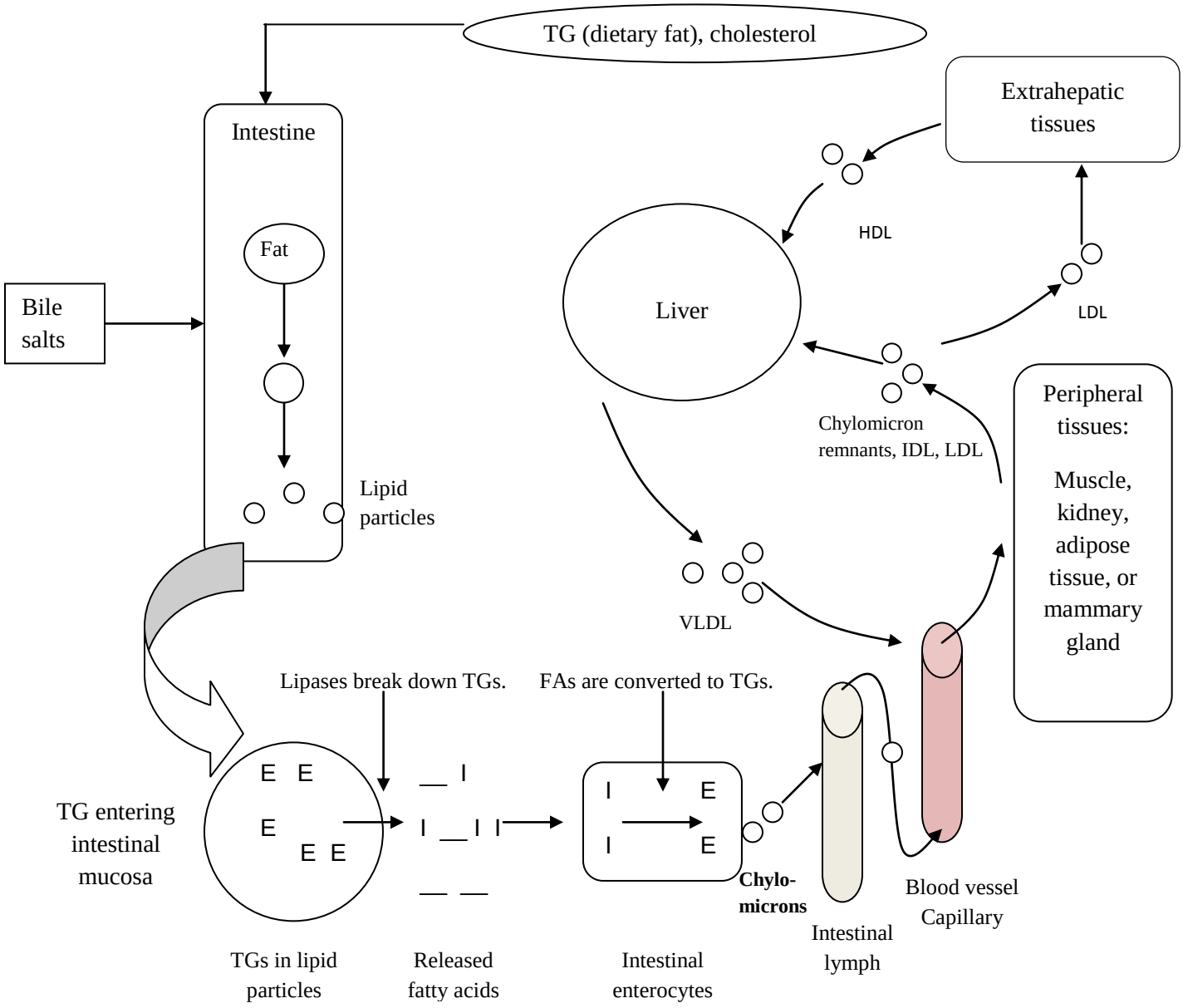


Figure 2.1. Absorption and transport of TG. TGs are hydrolyzed in the small intestine, transported into enterocytes, packaged into chylomicrons, and transferred to tissues for fuels, storage and other purposes. Excessive TGs in the liver are transported in VLDLs to adipose and muscle tissue through the blood vessels. Lipoprotein lipase in the vessels hydrolyzes TGs in chylomicrons and VLDLs to release free fatty acids. Chylomicrons thus become chylomicron remnants, and VLDLs become IDLs and LDLs. Remnants, IDLs and LDLs finally move to the liver. Some LDLs are delivered to extrahepatic tissues, which can secrete HDLs containing TGs and cholesterol in the liver. "E" in the lipid particle resembles the TGs with three fatty acids attached to the glycerol backbone. "I" resembles the released fatty acids from TGs.

TGs and other lipids are combined with an apolipoprotein ApoB48, and packaged into lipoprotein complexes called chylomicrons. Chylomicrons are secreted to lymph and enter into systemic circulation. In the capillaries in adipose tissues, TGs contained in chylomicrons are hydrolyzed into glycerol and fatty acids by lipoprotein lipase. In the process of releasing TGs, chylomicrons become chylomicron remnants and finally travel to the liver. The hydrolyzed fatty acids are taken up by adipocytes and incorporated into TGs for storage.

Excessive TGs in the liver are also packaged into very low-density lipoprotein (VLDL). The TG-rich VLDLs then transport TGs to adipose tissue for storage. By giving up TG, VLDL becomes the intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL), and is finally transported back to the liver. The weight percentages of TG in chylomicrons, VLDLs, IDLs and LDLs are 85%, 50%, 31% and 10%, respectively. Besides, the liver, the small intestine also secrete high-density lipoprotein (HDL) precursors to enable the formation of HDL by extrahepatic tissues. The resultant HDLs are transported back to the liver to recycle cholesterol and a relatively small amount of TGs from other tissues. The weight percentage of TG in HDLs is about 4%.

When animals undergo vigorous physical activity, starvation or fasting, glucose from glycogen is rapidly used up. TGs stored in adipocytes are then broken down to fatty acids by hormone sensitive lipase. Fatty acids are released into blood, bound to serum albumin followed by transport to various tissues, such as the liver, muscles and kidneys (Figure 2.2). After arriving at these tissues, fatty acids are detached from albumin to translocate into cells by transporter proteins in the plasma membrane. The brain cannot utilize fatty acids directly and high levels of plasma fatty acids are toxic, so circulating fatty acids are converted to ketone bodies by the liver to serve as metabolic fuel for β-oxidation. Besides dietary fats, TGs can also be derived *de novo* from glucose. The biosynthesis of TGs not only conserves the energy contained in glucose, but also serves important physiological functions.

Both high serum levels of fatty acids or glucose can lead to reactive oxygen species and insulin resistance (Inoguchi *et al*., 2000; Groop *et al*., 1989). Thus, excessive glucose needs to be incorporated into fatty acids, which are then incorporated into TG to maintain plasma glucose and fatty acid levels (Unger and Grudy, 1985).

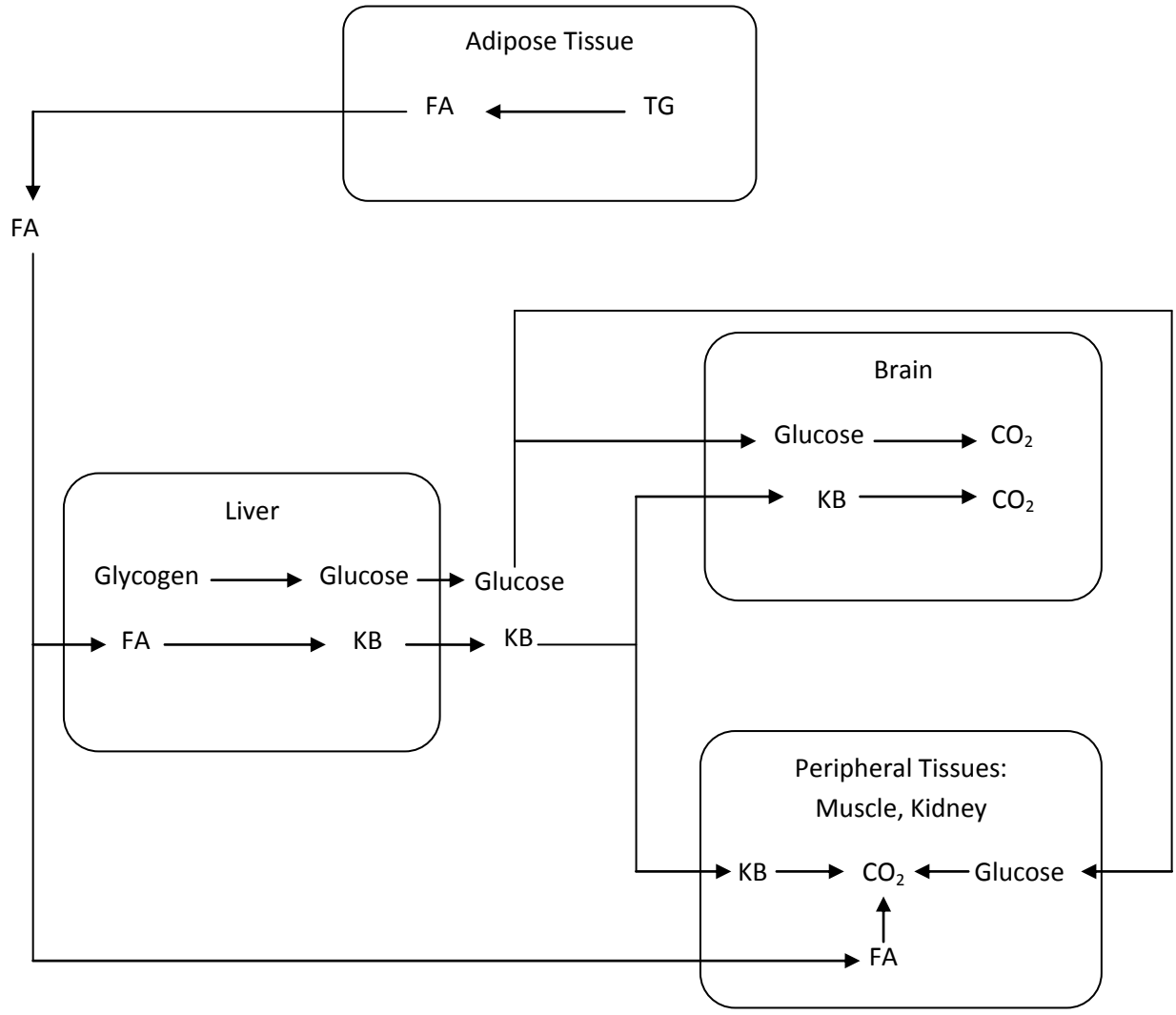


Figure 2.2. The utilization of TG under nutritional stress. Glycogen is broken down to supply glucose under the fasting condition. When glycogen is used up, TG in adipose tissue is then broken down into FA and transported to other tissues as the fuel. The brain cannot use FA, so a part of FAs are converted into ketone bodies (KB) for energy supply.

Excessive accumulation of TGs in adipose tissue, liver, heart and pancreatic beta cells is associated with obesity, insulin resistance and type II diabetes mellitus in humans (Unger, 2002). The prevalence of obesity has increased remarkably over the last twenty years. In Canada, ~65% of Canadian adults are considered overweight and ~25% are obese (Tjepkema *et al*., 2009). Obese individuals are at risk for diabetes and cardiovascular disease. To better prevent obesity and obesity-associated disorders, an understanding of TG biosynthesis is of biomedical importance.

2.2 Triacylglycerol Biosynthesis and Acyl-CoA:Diacylglycerol Acyltransferase (DGAT)

TG biosynthesis occurs via either the monoacylglycerol pathway in the intestine or glycerol phosphate pathway (*de novo* synthesis, also called Kennedy pathway) (Figure 2.3) (Kennedy, 1957; Bell and Coleman, 1980; Lehner and Kuksis, 1996). For the monoacylglycerol pathway, water-insoluble dietary fats have to be emulsified and hydrolyzed to 2-monoacylglycerol and fatty acids prior to passing through the intestinal lumen. Monoacylglycerol and fatty acids are re-synthesized by MGAT or DGAT1 into 1,2-diacylglycerol that are subsequently converted into TGs by DGAT, and packaged into chylomicrons for the distribution of TG to tissues. For the *de novo* synthesis of TG, the source of fatty acids and glycerol backbone can be either synthesized by the cell itself or from diet directly.

To initiate TG biosynthesis, fatty acids have to be activated by acyl-CoA synthetase into their acyl-CoA derivatives. Glycerol-3-phosphate (G3P) is then esterified with acyl-CoA by glycerol phosphate acyl-CoA acyltransferase (GPAT) to yield lysophosphatidate (LPA) (Figure 2.3). The second acylation reaction is catalyzed by lysophosphatidate acyltransferase (LPAT) to generate phosphatidate (PA) from LPA. Next, the phosphate group of PA is hydrolyzed by phosphatidate phosphatase (PAP) to generate 1,2-diacylglycerol, which is used by DGAT with a third acyl-CoA to generate the TG (Figure 2.4A) (Coleman and Lee, 2004; Vance and Brindley, 1991). In addition, diacylglycerol can also be used to synthesize phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Specifically, phosphatidylcholine is different from phosphatidylethanolamine by containing additional three methyl groups in its amino group. GPAT localizes in both ER and mitochondria, LPAT localizes in ER and PAP is present in the cytosol and plasma membrane (Coleman and Lee, 2004). When cells are loaded with fatty acids cytosolic, PAP translocates to the ER membrane. Two DGAT enzymes have been identified, DGAT1 and DGAT2. Both DGAT1 and DGAT2 are expressed at high levels in tissues active in TG synthesis, such as white adipose tissue, small intestine, mammary gland and liver. DGAT1 and DGAT2 are very different enzymes and are encoded by unrelated genes that share no sequence similarity and have different physiological roles (Yen *et al.,* 2008). DGAT2 localizes to ER and mitochondria, while DGAT1 and LPAT are only found in ER.

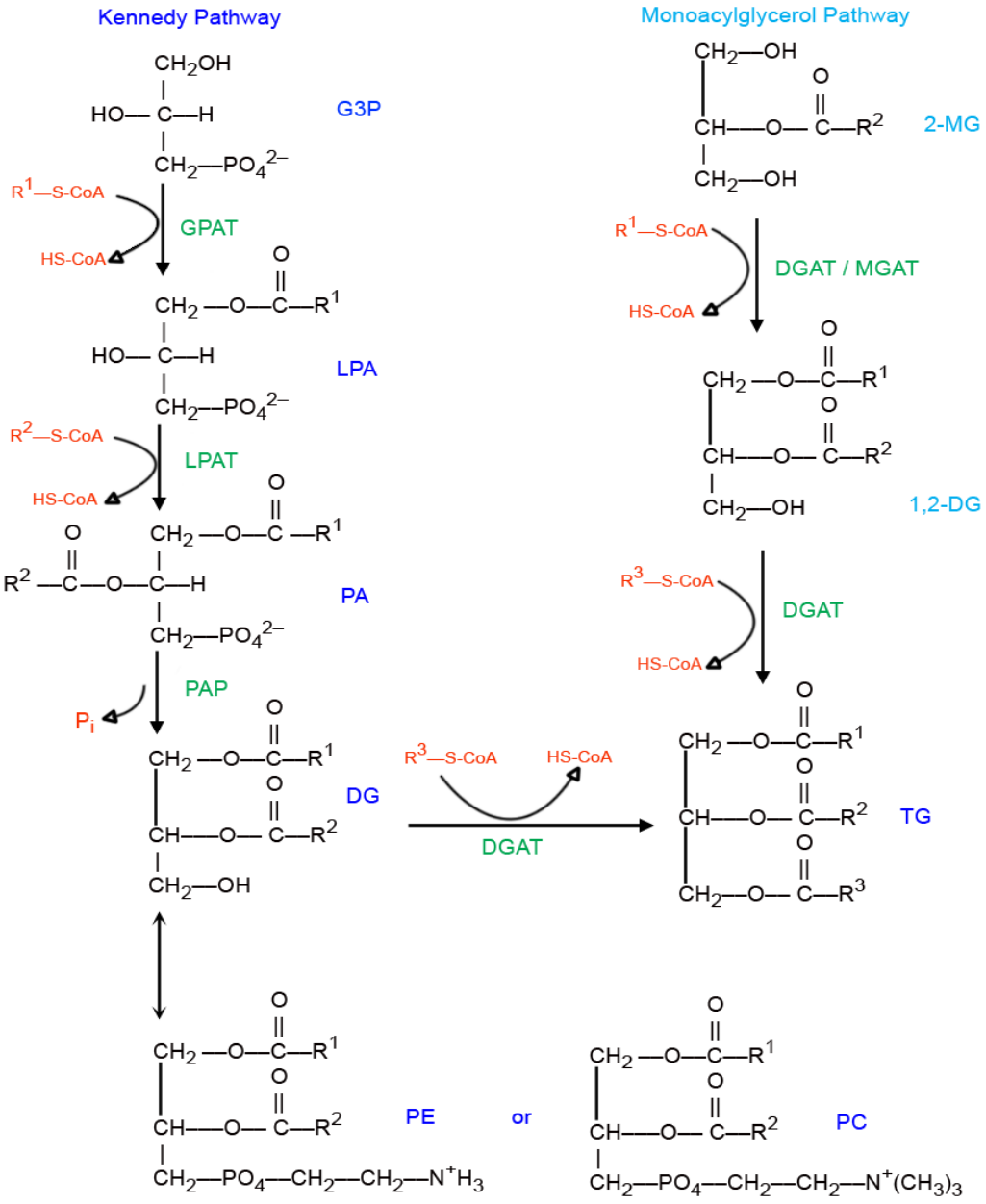


Figure 2.3. TG synthetic pathways. Major key enzymes in TG synthetic pathways and their substrates and products. G3P, glycerol 3-phosphate. GPAT, glycerol 3-phosphate acyltransferase. LPA, lysophosphatidate. LPAT, acyl-CoA:lysophosphatidate acyltransferase. PAP, phosphatidate phosphatase. DGAT, acyl-CoA:diacylglycerol acyltransferase. PA, phosphatidate. PE, phosphatidylethanolamine. PC, phosphatidylcholine. TG, triacylglycerol. DG, diacylglycerol. MG, monoacylglycerol. MGAT, acyl-CoA:monoacylglycerol acyltransferase.

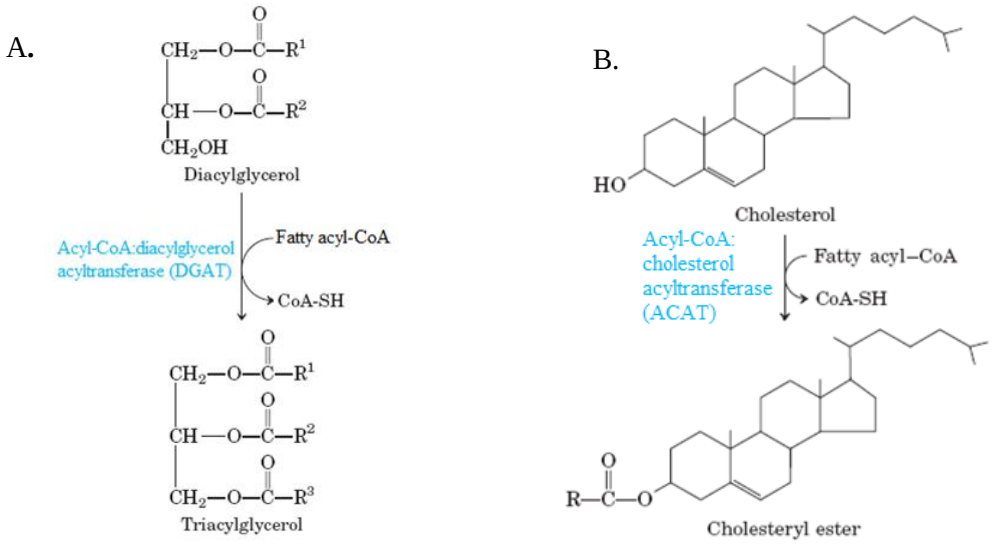


Figure 2.4. Biosyntheses of TG and cholesterol ester. (A) Diacylglycerol is esterified with fatty acyl-CoA by DGAT enzymes to form a triacylglycerol. (B) Cholesterol is esterified with fatty acyl-CoA by ACAT.

2.3 DGAT1

2.3.1 DGAT1 Gene Family

Although the TG biosynthetic pathway and DGAT activity were described in the 1950s by Kennedy *et al.* (Kennedy, 1957), the gene for DGAT1 has only been recently identified due to its homology to acyl-CoA:cholesterol acyltransferase (ACAT) enzymes (Cases *et al.*, 1998a). ACAT enzymes catalyze the formation of cholesterol esters (CE) from cholesterol and fatty acyl-CoA (Figure 2.4B). Murine DGAT1 has ~20% sequence identity relative to murine ACAT1, mostly at the C-terminus. DGAT1 orthologs have also been identified in many different species. DGAT1 is a member of the membrane-bound O-acyltransferase protein family (MBOAT) (Hofmann, 2000). MBOAT protein members catalyze *O*-acylation reactions that transfer fatty acyl-CoA onto the hydroxyl or thiol groups of lipid and protein acceptors.

2.3.2 Structure and Biochemistry of DGAT1

DGAT1 from mammalian species contains approximately 500 amino acids (Cases *et al*., 1998a). It is an integral membrane protein localized to the ER and found capable of existing in oligomeric forms such as a dimer and tetramer (Cheng *et al*., 2001). In a recent study, murine DGAT1 was found to contain three transmembrane domains with the N-terminus oriented toward the cytoplasm and the C-terminus containing the active site projecting into the ER lumen (McFie *et al*., 2010) (Figure 2.5).

The N-terminus of DGAT1 appears to be required for dimer/tetramer formation (Weselake *et al*., 2006; McFie *et al*., 2010). It is not clear whether a higher order structure of DGAT1 is functionally significant for its catalytic activity. Cross-linking experiments showed that the active form of DGAT1 might be a dimer, and the deletion of amino acids 37-84 prevents tetramer formation and increased DGAT activity seven-fold. In the C-terminus, His-426 of DGAT1 was shown to be essential for enzymatic activity and to be part of the active site.

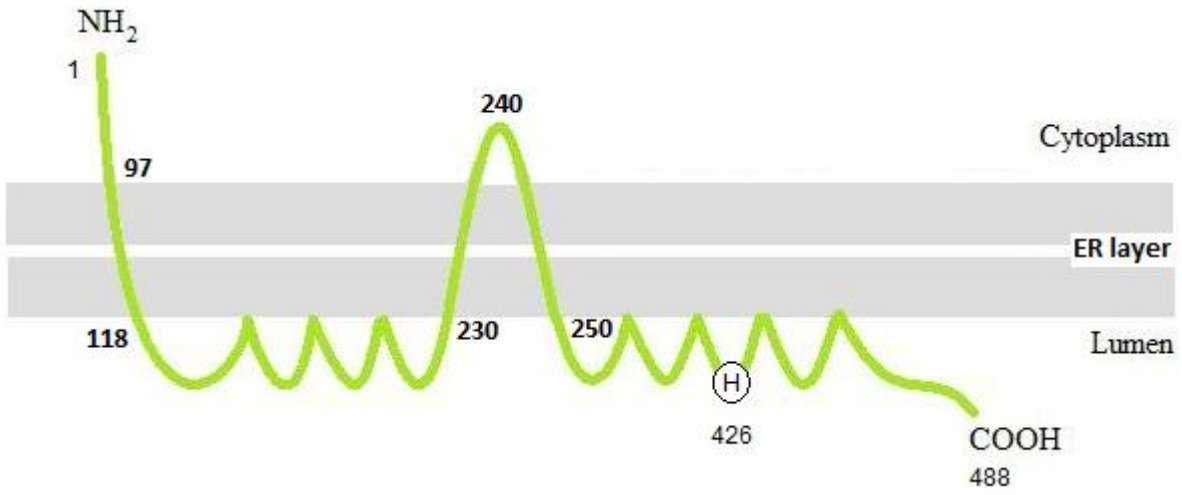


Figure 2.5. Model of the murine DGAT1 membrane topology. DGAT1 contains three transmembrane domains. Its N-termini faces the cytosolic side of ER and its C-termini faces the lumen side of ER. The circle with H letter indicates the active site histidine at position 426. The cytosol and ER lumen sides are indicated. The first transmembrane domain of DGAT1 extends from amino acid 97 to 118. The second and third transmembrane domains of DGAT1 extend from 230-240 and 240-250.

Human ACAT1 and ACAT2 also have highly conserved histidine residues in their active site regions (Guo *et al*., 2005; Das *et al*., 2008). Since the active site of DGAT1 was shown to be present in the ER lumen, it is possible that TG produced by DGAT1 could be channeled to nascent lipoproteins. However, the functional role of DGAT1 in the secretion of TG-rich VLDL is unclear. Some studies have shown a positive correlation between DGAT1 overexpression and increased VLDL secretion from the liver (Millar *et al*., 2006; Yamazaki *et al*., 2005; Liang *et al*., 2004), while a separate study showed that DGAT1 knockdown did not affect plasma VLDL levels in mice (Liu *et al*., 2008).

The substrate specificity of DGAT1 has not been carefully examined. It appears that DGAT1 prefers monounsaturated fatty acyl-CoA, such as oleoyl-CoA (18:1) as a substrate. There is also a preference for diacylglycerol containing monounsaturated fatty acids as well (Cases *et al*., 2001; Hiramine and Tanabe, 2011). Apart from DGAT activity, DGAT1 has additional acyl-CoA dependent acyltransferase activities, including acyl-CoA:retinol acyltransferase (ARAT) activity, acyl-CoA:monoacylglycerol acyltransferase (MGAT) activity and wax synthase activity (Yen *et al*., 2005). MGAT activity is capable of esterifying a monoacylglycerol with one fatty acyl-CoA to generate diacylglycerol. DGAT1 prefers 2-monoacylglycerol over 3- monoacylglycerol as a substrate to synthesize 1,2- diacylglycerol, the preferred substrate for DGAT1 and DGAT2 (Hiramine and Tanabe, 2011). This function of DGAT1 is particularly favored in the small intestine where the high activity events of TG hydrolysis and re-esterification occur. When rats were orally fed with a DGAT1 inhibitor XP620 that specifically suppressed DGAT1's MGAT activity, lipid absorption through the intestine was reduced ~50% (Cheng *et al*., 2008). However, DGAT1 was not essential for quantitative dietary TG absorption. DGAT1-deficient mice absorbed the same amount of dietary TG as wild-type mice, although they secreted TG into the circulation at a reduced rate (Buhman *et al*., 2002). Other enzymes such as DGAT2 and diacylglycerol transacylase were suggested to compensate for the lack of DGAT1 (Buhman *et al*., 2002). More interestingly, DGAT1-deficient mice specially designed only to express DGAT1 in the small intestine were shown to be susceptible to a high-fat diet induced hepatic steatosis and obesity (Lee *et al*., 2010). This indicated that DGAT1 was the preferential protein to synthesize TG and as well as stimulating TG secretion from enterocytes.

Another activity of DGAT1, ARAT activity, catalyzes the formation of retinyl esters from retinol (vitamin A) and fatty acyl-CoA. DGAT1 might function in assisting the absorption of vitamin A in intestine by converting it into retinyl esters that are incorporated into chylomicrons. Studies reported that *Dgat1*$^{-/-}$ mice have markedly reduced ARAT activities in liver and adrenal gland even though there was another enzyme, lecithin:retinol acyltransferase, (LRAT) considered to be involved in bulk retinyl ester synthesis (Orland *et al*., 2005; Ball *et al*., 1985). Retinol is the precursor of retinoic acid, a hormone that has a role in sebaceous gland function, hair growth and proliferation of mammary gland epithelial cells. *Dgat1*$^{-/-}$ mice showed characteristics of dry fur and hair loss due to lack of retinol (Chen *et al*., 2002). DGAT1 also uses fatty alcohols and fatty acyl-CoA to form wax monoesters and diesters (Yen *et al*., 2005). Wax esters coat the skin surface of mammals to prevent water loss and abrasion (Thody and Shuster, 1989). *Dgat1*$^{-/-}$ mice showed hair loss due to atrophy of the sebaceous glands, caused by a lack of wax diesters (Chen *et al.*, 2002a).

2.3.3 Physiological Roles of DGAT1

DGAT1 is expressed ubiquitously in mice and humans, in organs such as small intestine, liver, adipose tissue and mammary gland (Cases *et al*., 1998a). DGAT1's physiological roles were revealed by the disruption of DGAT1 expression in mice.

The mRNA level of DGAT1 directly correlated with fat storage in mice, as shown by mice of different genetic backgrounds (*Dgat1*$^{-/-}$, *Dgat1*$^{+/-}$, *Dgat1*$^{+/+}$) (Chen and Farese, 2005). In *Dgat1*$^{-/-}$ mice, the DGAT1 mRNA was not detectable and the tissue DGAT activity was significantly reduced in various tissues, such as adipose tissue, small intestine, liver, testes and brain (Smith *et al*., 2000). *Dgat1*$^{-/-}$ mice were leaner and showed less adipose mass (~50% reduction), improved insulin sensitivity and glucose metabolism compared to wild-type mice (Smith *et al*., 2000; Tsai *et al*., 2007). TG levels in liver as well as adrenal glands decreased moderately in *Dgat1*$^{-/-}$ mice. *Dgat1*$^{-/-}$ mice were also resistant to diet-induced obesity and showed a decreased rate of TG absorption when challenged by a high-fat diet. Although *Dgat1*$^{-/-}$ mice exhibited reduced white adipose tissue, the fatty acid composition of adipose tissue was similar between *Dgat1*$^{-/-}$ mice and wild-type mice. In addition, fasting serum TG levels in *Dgat1*$^{-/-}$ mice

were similar to those in wild-type mice. This indicated that quantitatively, the disruption of DGAT1 did not affect the TG secretion from livers.

As a matter of fact, in *Dgat1*$^{-/-}$ mice hepatic TG levels were similar with those in wild-type mice with chow-fed diet (Villanueva *et al*., 2009). After high-fat feeding, *Dgat1*$^{-/-}$ mice showed ~80% decrease in hepatic TG levels compared to wild-type mice. In the mice with liver-specific knockout of DGAT1, hepatic TG levels also showed ~50% decrease. These facts indicated that DGAT1 disruption could reduce TG storage in the liver when challenged with high-fat diet, even though DGAT2 is also expressed in the liver.

DGAT1 deficiency also has profound effects on other tissues, such as mammary glands, skin. Female *Dgat1*$^{-/-}$ mice could not produce milk, and few lipid droplets were found in their mammary tissues (Smith *et al*., 2000). Hence, DGAT1 is essential for lactation. *Dgat1*$^{-/-}$ mice also displayed fur abnormalities such as dryness and hair loss. This resulted from that abnormal sebaceous glands of *Dgat1*$^{-/-}$ mice produce little type II wax diesters, could not maintain moisture and protect the fur of mice. The disruption of DGAT1 did not result in the malabsorption of TG in the intestine or abnormalities in chylomicron synthesis (Buhman *et al*., 2002). *Dgat1*$^{-/-}$ mice showed a normal quantitatively dietary TG absorption, but the small intestine secreted TGs into the bloodstream at a lower rate compared to wild-type mice.

2.4 DGAT2

2.4.1 DGAT2 Gene Family

Because *Dgat1*$^{-/-}$ mice still have abundant TG and DGAT activity, it was thought that there must be another DGAT gene. In 2001, three years after the discovery of DGAT1, a second DGAT, DGAT2, was identified (Lardizabal *et al*., 2001; Cases *et al*., 2001). DGAT2 is in the DGAT2/MGAT gene family, which includes MGAT1, MGAT2, MGAT3 and wax monoester synthases. The protein sequence of DGAT2 shares 40-50% identity with MGAT enzymes and wax synthase, however, DGAT2 does not have MGAT or wax synthase activity. All DGAT2 protein family members contain a highly conserved HPHG amino acid sequence.

2.4.2 Structure and Biochemistry of DGAT2

In most species, DGAT2 contains 350-400 amino acids and is 42 kDa (Cases *et al*., 2001). DGAT2 localizes to the ER, and is less hydrophobic compared to DGAT1 according to Kyte-Doolittle hydrophobicity analysis. DGAT2 has also been found on the surface of cytosolic lipid droplets, mitochondria and mitochondria-associated-membranes (MAM) that are the specialized domains of the ER associating tightly with mitochondria (Kuerschner *et al*., 2008; Stone *et al*., 2009). This juxtaposition of ER membrane and mitochondrial membranes is thought to channel lipids between ER and mitochondria. The fact that DGAT2 could redistribute around lipid droplets suggested that TG synthesized by DGAT2 could be directed into cytosolic lipid droplets for storage.

DGAT2 appears to be more active at lower concentration of substrates (fatty acyl-CoA, 0-50 μM), unlike DGAT1 (with higher activity at >100 μM), indicating DGAT2 has a higher affinity for fatty acyl-CoA. One study reported that DGAT2 appeared to utilize fatty acids from *de novo* synthesis, since stearoyl-CoA desaturase 1 (SCD1) deficiency that disrupted substrate channeling to DGAT2 decreased TG synthesis (Man *et al*., 2006). DGAT2 from *M. ramanniana* (oleaginous fungus) shows higher activities with short- and medium-chain fatty acyl moieties (6:0, 8:0, and 10:0) rather than longer chains (Lardizabal *et al*., 2001).

The topology of murine DGAT2 has been determined. DGAT2 is an integral membrane protein with two transmembrane domains and both N- and C-termini facing toward the cytosolic side of the ER (Figure 2.6) (Stone *et al*., 2006). The first transmembrane domain (TMD1) of DGAT2 is between amino acids 66-88, and the second one (TMD2) is between amino acids 93-115 with the short loop between them oriented towards the ER lumen. TMD1 contains an ER targeting signal for DGAT2 localization to the ER (McFie *et al*., 2011). A DGAT2 mutant lacking both transmembrane domains did not localize to the ER and instead was localized to mitochondria. Amino acids 61-66 of DGAT2, which are exposed to the cytosol, are a mitochondrial targeting signal sequence. The reason for the interaction of DGAT2 with mitochondria is not clear (Stone *et al*., 2009). TMD1 also contains a conserved neutral lipid-binding domain (amino acids 80-87, FLVLGVAC) that is also present in other enzymes involved in lipid metabolism such as LPAT and cholesterol ester transfer protein (CETP) (Stone *et al*., 2006; Au-Young and Fielding, 1992).

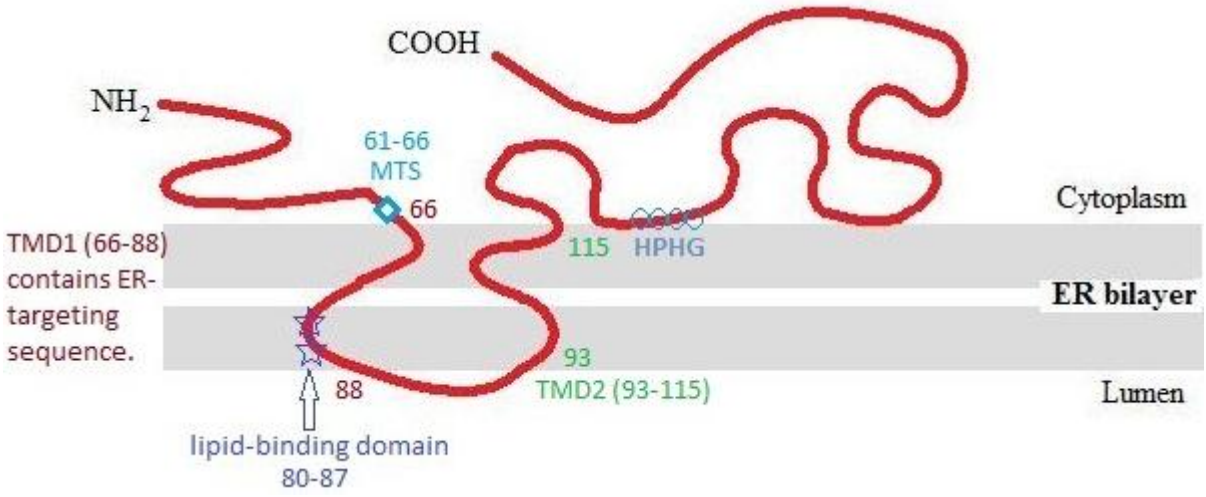


Figure 2.6. Model of the murine DGAT2 membrane topology. DGAT2 contains two transmembrane domains. The short loop between the two transmembrane domains may be either exposed to the cytosol or reside in the ER bilayer. The mitochondria-targeting sequence (MTS) is in the region of amino acids 61-66. The first transmembrane domain (TMD1) is between amino acids 66-88 and contains an ER-targeting sequence. The lipid-binding domain is between amino acids 80-87 and is indicated with two stars. The second transmembrane domain (TMD2) is between amino acids 93-115. The conserved HPHG sequence is labeled as circles.

This domain serves to bind or transfer neutral lipids. A mutation in this domain of DGAT2 resulted in a large decrease of DGAT activity in *in vitro* assays, indicating that this domain is important for the enzymatic activity of DGAT2 (Stone *et al*., 2006).

The cytoplasmic C-terminus of DGAT2 contains the active site histidine in the highly conserved HPHG sequence. The enzymatic activity of DGAT2 is in the cytosolic side of the ER. This suggests that TG synthesized by DGAT2 would be released at the cytosolic side of the ER and ready to translocate into lipid droplets for storage instead of being secreted. In support of this, the C-terminus of DGAT2 is indicated to have a role in directing DGAT2 to lipid droplets (McFie *et al*., 2011). When the C-terminus of DGAT2 was truncated or mutated (232-250 or 327-350), DGAT2 failed to co-localize with lipid droplets.

2.4.3 Physiological Roles of DGAT2

DGAT2 is expressed in most mouse/human tissues, but is highly expressed in white adipose tissue. DGAT2 plays a very different functional role from DGAT1 (Stone *et al*., 2004).

DGAT2 is more a potent enzyme for bulk TG synthesis. DGAT2 overexpression leads to more TG production than does DGAT1 in rat hepatoma cells. In yeast, DGAT2 accounts for the majority of TG produced (Sorger and Daum, 2002). From DGAT2 deficient (*Dgat2*$^{-/-}$) mice, the functional roles of DGAT2 are well illustrated (Stone *et al*., 2004). *Dgat2*$^{-/-}$ mice showed great decrease in size of adipose tissue and TG storage (~90%). Also, *Dgat2*$^{-/-}$ mice had a defective skin permeability barrier causing rapid dehydration. As a result, *Dgat2*$^{-/-}$ mice did not survive long after birth (only 6-8 h). In contrast, plasma TG levels and susceptibility to diet-induced obesity in *Dgat2*$^{+/-}$ mice were similar to those of wild-type mice (Chen and Farese, 2005). DGAT2 is therefore the major DGAT enzyme responsible for bulk TG synthesis.

Moreover, DGAT2 displayed a protective role in preventing lipotoxicity where excessive fatty acids impair the insulin signaling pathway (Unger, 2002). Enlarged TG pools usually accompanied more free fatty acids and fatty acid-derived metabolites, causing higher oxidant stress, fibrosis and hepatocyte necrosis. The overexpression of DGAT2 in the liver of transgenic mice led to hepatic steatosis with increased TG, diacylglycerol, ceramides and fatty acid but no insulin resistance (Monetti *et al*., 2007). In a later study, hepatic DGAT2 overexpression in mouse liver did result in insulin resistance of the liver (Jornayvaz *et al*., 2011). The latter study appears more plausible since the mice in the earlier study were tested 3 days postoperatively and latter study gave the mice 6-7 days to recover before further tests. Therefore, the first study did not leave enough time for the physiological function of the liver in mice to become fully recovered. In contrast, chronically suppression of DGAT2 expression using an anti-sense oligonucleotide caused hepatic fatty acid levels to increase. In this case, although the liver was protected from hepatic steatosis, it seemed that more liver damage, such as fibrosis, was caused from a high fatty acid level and resultant oxidative stress (Yamaguchi *et al*., 2007). Because the disruption of DGAT2 was lethal to mice, a clearly identified physiological role of DGAT2 could not be further addressed.

2.5 Regulation of DGAT Enzymes

In the TG biosynthetic pathway, the conversion of glycerol-3-phosphate into lysophosphatidate is considered rate-limiting (Dricks and Sul, 1997). However, since DGAT1 and DGAT2 are at a branch point of the glycerol-3-phosphate pathway, regulation of these

enzymes may determine if substrates are used for TG or phospholipid biosynthesis (Figure 2.3) (Bagnato and Igal, 2003). The regulation of DGAT enzymes can occur at several levels.

DGAT1 and DGAT2 expression can be regulated by nutritional and physiological status. For instance, free fatty acid release by epinephrine rosiglitazone stimulation can increase DGAT1 mRNA levels in 3T3-L1 adipocytes (Liu *et al*. 2008). On the other hand, the insufficiency of fatty acids could decrease DGAT2 mRNA levels when mice undergo fasting, DGAT2 mRNA levels in adipose tissue and liver decreased (Meegalla *et al*., 2002; Chen *et al*., 2002b). Importantly, DGAT1 and DGAT2 expression could be regulated collaboratively or individually. During adipocyte differentiation, mRNA levels of DGAT1 and DGAT2 increased remarkably with a corresponding increase in DGAT activity (Ludwig *et al*., 2002; Ranganathan *et al*., 2006; Payne *et al*., 2007). In the case of individual regulation, glucose was suggested to preferentially enhance DGAT1 mRNA levels instead of DGAT2, whereas insulin specifically increased DGAT2 mRNA levels (Meegalla *et al*., 2002). Hypertrophy of adipocytes was associated with attenuated leptin action and increased DGAT2 mRNA levels and down-regulated DGAT1 mRNA levels in the adipocytes of *Irs2*$^{-/-}$ mice (insulin receptor substrate, Irs; insulin resistant mice), leptin-deficient ob/ob mice (obesity-inducible mice) and wild type mice (Suzuki *et al*., 2005).

Specifically, the regulation of DGAT1 and DGAT2 expression was through the MEK-ERK signaling pathway (Wang *et al*., 2010; Au *et al*., 2003). When this signaling pathway was inhibited, both DGAT1 and DGAT2 mRNA levels were up-regulated. In addition, transcription factors, such as C/EBPα or C/EBPβ, also regulated DGAT1 and DGAT2 mRNA levels (Payne *et al*., 2007). Specifically, increased CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ levels could induce C/EBPα and peroxisome proliferator-activated receptor gamma (PPARγ), and the latter activated DGAT1 and DGAT2 expression.

DGAT enzymes can also be regulated post-transcriptionally (Yu *et al*., 2002a; Casaschi *et al*., 2004; Waterman *et al*., 2002). In mature adipocytes (3T3-L1) overexpressing human DGAT1, a 7-fold increase in DGAT1 mRNA corresponded to a 90-fold increase in DGAT1 protein. In contrast, forced DGAT1 expression in preadipocytes resulted in a 20-fold increase in DGAT1 mRNA only resulted in 2.6-fold increase in DGAT1 protein. This observation suggested that the post transcriptional regulation of DGAT1 decides both its enzyme activity and

intracellular TG accumulation. Though the mechanism of post-transcriptional control is not well understood, several phosphorylation events by cAMP-dependent or Ca^{2+} calmodulin-dependent protein kinases were shown to enhance DGAT activity about 20-fold in parotid gland cells from guinea pigs (Soling *et al*., 1989; Maziere *et al*., 1986). Protein kinase A is a known cAMP-dependent protein kinase, and our lab has observed that protein kinase A (PKA) inhibits DGAT1, likely by phosphorylating the protein (unpublished observation, S. Stone and R. Farese). Like protein kinase A, protein kinase C is also a Ser/Thr kinase, and DGAT2 contains a protein kinase C (PKC) phosphorylation site that localizes in a glycerol phospholipid domain involved in the active site (TL, 301-302) of the acyltransferase reaction (Cases *et al.*, 2001). Therefore, the regulation by phosphorylation of Ser/Thr sites in DGAT enzymes might be important for the control of DGAT activity.

Besides Ser/Thr phosphorylation sites, a tyrosine protein kinase phosphorylation site is also thought to regulate DGAT activity. One conserved tyrosine phosphorylation site (316) in human DGAT1 was proposed to regulate the enzymatic activity of DGAT1. However, a later experiment showed that the mutagenesis of the tyrosine site did not significantly change the activity of the enzyme or overall rates of TG accumulation (Lau and Rodrigeuz, 1996; Yu *et al*., 2002a). In contrast, the mutation of the tyrosine phosphorylation site (392) in DGAT1 from Indian cress (*Tropaeolum majus*) caused 80-100% loss of DGAT activity (Xu *et al*., 2008). Taken together, it remains unclear that whether DGAT activity is regulated by phosphorylation of tyrosine 392.

DGAT activity can be regulated by inhibitors as well (Casaschi *et al*., 2004; Ganji *et al*., 2004). When liver hepatocellular carcinoma (HepG2) cells were treated with taxifolin, a plant flavonoid (a non-competitive DGAT inhibitor), DGAT activity and TG synthesis from microsomes significantly decreased (35% and 37%, respectively). However, DGAT1 and DGAT2 mRNA levels remained constant. Also, niacin (DGAT2 inhibitor) decreased DGAT activity of DGAT2 in a non-competitive manner without affecting DGAT2 mRNA levels (Ganji *et al*., 2004). Substrate concentration also influences DGAT activity of DGAT enzymes. High concentration of oleoyl-CoA (>10 μM) and 1,2-dioleoylglycerol (>125 μM) was noted to inhibit DGAT activity (Ganji *et al*., 2004). In addition, 2-bromopalmitoyl-CoA inhibited DGAT activity by non-competitive inhibition (Coleman *et al*., 1992).

2.6 Triacylglycerol Synthesis Protein Complex

Many proteins form complexes in order to promote the efficient synthesis of products. Therefore, it is reasonable that DGAT1 and DGAT2 interact with other proteins as part of a TG-synthetase protein complex. For instance, DGAT from castor bean was suggested to associate with acyl-CoA binding proteins as it contained a signature domain (219-247) that was common for such binding interactions in bacteria (He *et al*., 2004). Two proteins have been identified that interact with DGAT2. Tung tree (*Vernicia fordii*) glycerol 3-phosphate acyltransferase 8 (GPAT8) was shown to interact with tung tree DGAT2 (Gidda *et al*., 2011). The other protein that interacts with DGAT2 is SCD1. SCD1 converts saturated fatty acids into mono-unsaturated fatty acids and directs mono-unsaturated fatty acids to DGAT2 (Man *et al*., 2006). Overexpression of SCD1 increased TG synthesis and the percentage of mono-unsaturated fatty acids in TGs (Listenberger *et al*., 2003).

Moreover, two TG synthetase complexes have been proposed in the literature (Lehner and Kuksis, 1995; Gangar *et al*., 2001). From detergent-solubilized rat intestinal enterocytes, a TG-synthesis complex was obtained upon Cibacron blue 3GA-agarose affinity chromatography (Lehner and Kuksis, 1995). After the purification, the complex was tested and found to possess acyl-CoA ligase, acyl-CoA acyltransferase (AAT), MGAT, and DGAT activities. This study also showed that acyl-CoA acyltransferase antibodies inhibited TG synthesis and suggested that AAT was responsible for transferring the fatty acyl moieties to DGAT. In support of this, the removal of diacylglycerol acceptors led to fatty acyl moieties releasing to water in an *in vitro* assay. Although the sequence of AAT has never been identified, immunoprecipitation of AAT activities caused a decrease in MGAT and DGAT activities. This finding suggested that AAT was part of a complex catalyzing TG synthesis. Therefore, the sequential order for acyl-moiety transfer was suggested to be as shown in Figure 2.7. Acyl-CoA ligase activates fatty acids into acyl-CoA by consuming one molecule of ATP. The resulting acyl-CoA is transferred by AAT to DGAT, which converts diacylglycerol to TG.

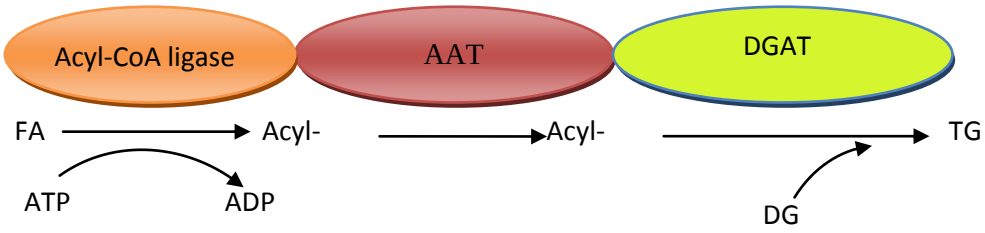


Figure 2.7. Sequential order for acyl-CoA transfer. FA is channeled through the protein complex for TG synthesis. AAT, acyl-CoA acyltransferase. FA, fatty acid.

In yeast (*Rhodotorula glutinis*), a cytosolic TG biosynthetic multienzyme complex was reported (Gangar *et al*., 2001). The soluble fraction of yeast cells was separated by SDS-PAGE, and gels containing TG synthase were excised and purified on a gel-filtration column. The eluted proteins were subjected to isoelectric focusing for determining polypeptide components and followed by activity assays. The result showed that the complex contained almost all of the main enzymes required for the *de novo* TG synthesis including LPAT, PAP and DGAT.

These findings provide strong evidence that DGAT enzymes are part of a protein complex or complexes to synthesize TG. Moreover, as DGAT1 and DGAT2 may be engaged in different cellular processes and conceivably have different protein partners. DGAT-interacting proteins might further determine dissimilar functional and physiological roles of DGAT1 and DGAT2.

2.7 Hypothesis and Rationale

Our hypothesis is that both DGAT1 and DGAT2 interact with other intracellular proteins and/or exist as part of a protein complex that facilitates the synthesis, storage and secretion of TG.

Rationale

TG synthesis and its incorporation into cytosolic lipid droplets or secretion as part of lipoproteins remain poorly characterized cellular events. Almost every cellular process requires protein-protein interactions. Therefore, it is very likely that DGAT1 and DGAT2 interact with

other proteins either constantly or transiently to facilitate the synthesis of TG and/or its storage and secretion.

2.8 Objectives

2.8.1 Identify DGAT1 and DGAT2 Interacting Proteins

DGAT1 and DGAT2 will be immunoprecipitated from cells. Interacting proteins bound to DGAT1 and DGAT2 will be identified by mass spectrometry.

2.8.2 Examine the Roles of DGAT1/DGAT2 Interacting Proteins in TG Synthesis, Storage and Secretion

Interacting proteins will be knocked down or overexpressed in cell culture. Their effects on DGAT activities and TG synthesis will be examined.

3. MATERIALS AND METHODS

3.1 Reagents

Analytical grade or higher reagents were used for all experiments. Names of reagents, suppliers and addresses of suppliers are listed in Table 3.1.

Table 3.1: List of Reagents and Suppliers

Reagent	**Supplier/address**
Common Reagents	
DNA restriction enzymes	NEB/Mississauga, Ontario, Canada
GeneRuler™ 1kb DNA ladder	Fermentas/Burlington, Ontario, Canada
6× DNA loading dye	Fermentas/Burlington, Ontario, Canada
T4 DNA Ligase	Invitrogen/Burlington, Ontario, Canada
CHAPS	Bio Basic/Markham, Ontario, Canada

Bacterial Culture Reagents	
DH5α Competent *Escherichia coli*	Invitrogen/Burlington, Ontario, Canada
NM522 Competent *Escherichia coli*	Stratagene/La Jolla, California, USA
XL 10-GOLD® Ultracompetent *Escherichia coli*	Stratagene/La Jolla, California, USA
Ampicillin	Bio Basic/Markham, Ontario, Canada

Zeocin	InvivoGen/San Diego, California, USA
Yeast Extract	EMD/Madison, Wisconsin, USA
Tryptone	Bio Basic/Markham, Ontario, Canada
NZ Amine (casein hydrolysate)	Sigma-Aldrich/Oakville, Ontario, Canada

Cell Culture Reagents	
Dulbecco's Modified Eagle's Medium – Low Glucose (D6046)	Sigma-Aldrich/Oakville, Ontario, Canada
Fetal Bovine Serum	PAA/Etobicoke, Ontario, Canada
Antibiotic-Antimycotic (100 ×) (Penicillin and streptomycin)	Invitrogen/Burlington, Ontario, Canada
Bovine Serum Albumin	Sigma-Aldrich/Oakville, Ontario, Canada
Oleic Acid	Sigma-Aldrich/Oakville, Ontario, Canada
Diacylglycerol	Sigma-Aldrich/Oakville, Ontario, Canada
Cholesterol	Sigma-Aldrich/Oakville, Ontario, Canada
Phosphatidylcholine	Sigma-Aldrich/Oakville, Ontario, Canada
Triton WR-1339	Sigma-Aldrich/Oakville, Ontario, Canada
[^{14}C]Oleoyl CoA	ARC/St. Louis, Missouri, USA
[^{3}H]Glycerol	ARC/St. Louis, Missouri, USA
0.05% Trypsin-EDTA	Invitrogen/Burlington, Ontario, Canada

Commercial Kits	
QIAprep® Spin Miniprep Kit	Qiagen/Mississauga, Ontario, Canada
QIAquick® Gel Extraction Kit	Qiagen/Mississauga, Ontario, Canada
QIAquick® PCR Purification Kit	Qiagen/Mississauga, Ontario, Canada
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific/Rockford, Ontario, Canada
Bio-Rad D_C Protein Assay Kit	Bio-Rad/Hercules, California, USA

Protein Analysis Reagents	
30% Acrylamide/Bis Solution (29:1)	Bio-Rad/Hercules, California, USA
PageRuler™ Prestained Protein ladder Plus (SM1811, SM0671)	Fermentas/Burlington, Ontario, Canada
Autoradiography Film	Deville Scientific/Metuchen, New Jersey, USA
3× FLAG® Peptide	Sigma-Aldrich/Oakville, Ontario, Canada
Monoclonal Anti-Flag M2	Sigma-Aldrich/Oakville, Ontario, Canada
Immobilized Protein A	Thermo Scientific/Rockford, Ontario, Canada

siRNA Transfection Reagents	
Opti-MEM® I Reduced Serum Medium	Invitrogen/Burlington, Ontario, Canada
siRNA oligonucleotides	Invitrogen/Burlington, Ontario, Canada
Lipofectamine™	Invitrogen/Burlington, Ontario, Canada

Table 3.2: List of Antibodies Used for Immunoblot Analysis

Name	Dilution	Supplier/Address
Mouse Anti-FLAG	1:4000	Sigma-Aldrich/Oakville, Ontario, Canada
Mouse Anti-ACAT1	1:1000	GenScript/Piscataway, New jersey, USA
Mouse Anti-Myc	1:50	9E10 hybridoma, Stone lab
Rabbit Anti-Myc	1:2000	Sigma-Aldrich/Oakville, Ontario, Canada

3.2 Bacterial Strains and Media Preparations

E. coli DH5α, NM522 and XL 10-GOLD® were used in this study for the propagation of plasmid DNA. They were grown in Luria-Bertani (LB) medium, which was prepared by adding 10 g tryptone, 5 g yeast extract, 10 g NaCl dissolved in 1 L double distilled water (ddH_2O). For LB agar plates, 15 g of agar was added to 1 L LB. Media was autoclaved for 20 minutes on the liquid cycle under 15 psi, and the cooled media was stored at 4 ˚C till used. If LB agar plates with antibiotics were required, antibiotics were added when the temperature of the LB cooled to ~55 °C, and media was poured into Petri dishes (~25 mL/100 mm plate). The concentration of ampicillin was 100 μg/mL, and Zeocin was 50 μg/mL.

NZY medium was prepared for *E. coli* XL 10-Gold® Ultracompetent cells. NZY medium contained 10 g NZ amine (casein hydrolysate), 5 g yeast extract, 5 g NaCl with deionized H_2O to a final volume of 1 L, and the pH of the medium was adjusted to 7.5 before autoclaving. Filter-sterilized supplements (final concentration: 0.02 M glucose, 12.5 mM $MgCl_2$ and 12.5 mM $MgSO_4$) were added prior to use.

Terrific Broth (TB) was used for the large-scale amplification of plasmid DNA. For 200 mL TB, 2.4 g tryptone, 4.8 g yeast extract and 0.8 mL glycerol were added to 180 mL ddH_2O. Media was autoclaved and after cooling was supplemented with 20 mL potassium-salt solution (0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) and the appropriate antibiotics.

3.3 Molecular Cloning

3.3.1 Transformation of Competent Bacterial Cells

E. coli DH5α and XL 10-Gold® ultracompetent bacteria were transformed according to the supplied protocol. For the transformation of *E. coli* NM522, cells were first thawed in a 37˚C water bath, and 100 µL of cell stock was aliquoted into an ice-cold 13 mL sterile polypropylene tube. 5-50 ng DNA was added to the bacteria and the mixture was kept on ice for 10-30 minutes. The transformation mixture was heat shocked at 42 ˚C for 2 minutes, put on ice for another 2 minutes and subsequently plated out onto LB plates with appropriate antibiotics. The plate was incubated at 37 ˚C overnight.

3.3.2 Small Scale Plasmid DNA Preparations

Small scale plasmid DNA was prepared by first inoculating single bacterial colonies of interest into 5 mL TB with appropriate antibiotics. Cultures were then grown overnight at 37 ˚C with vigorous shaking. Plasmid DNA was prepared by using a Miniprep kit (QIAprep) and following the manufacturer's protocol.

3.3.3 Large Scale Plasmid DNA Preparations

Single colonies were picked and inoculated into 5 mL TB overnight at 37 ˚C. The subsequent culture was then transferred into 200 mL TB to grow overnight at 37 ˚C with vigorous shaking. The extraction of plasmid DNA was as described by the protocol of Sambrook and Fritsch *et al.* (1989).

3.3.4 Plasmids

The following describes plasmids used in the research. LacZ, N-terminal FLAG-tagged murine DGAT1 (FL-DGAT1) and DGAT2 (FL-DGAT2) were cloned individually into the eukaryotic expression vector pcDNA3.1 (Invitrogen). FL-DGAT2 and Myc-DGAT1 were cloned

together into multiple cloning sites of eukaryotic expression vector pBudCE4.1. Human ACAT1 cDNA fragment was cloned into cloning sites (*BamH*I/*EcoR*I) of pcDNA3 (Invitrogen). These plasmids were generous gifts from Dr. Robert Farese, Jr. (Gladstone Institutes, San Francisco). Various FL-DGAT1 mutants, Δ1-37, Δ38-84 and Δ1-85, were generated in a previous study using FL-DGAT1 as a template (McFie *et al*., 2010).

pFLAG3 was constructed by inserting three tandem FLAG epitopes in multicloning sites of mammalian expression vector, pRc/CMV (Invitrogen). pMyc3 was created the same way by inserting three Myc epitopes. Both pFLAG3 and pMyc3 vectors were generous gifts from Dr. Deborah Anderson (University of Saskatchewan, Saskatchewan). All plasmids were sequenced to confirm the desired DNA sequence.

3.3.4.1 Construction of FAR1 Plasmids

A 1.5 kb fragment of human FAR1 containing the coding region was ligated into multiple cloning sites (*Nhe*I/*Apa*I) of pFLAG3 and pMyc3. This work was kindly assisted by Pam McFie. The following is the procedure of generating FAR1 plasmids. Human cDNA of FAR1 was kindly provided by Dr. David Russell (University of Texas Southwestern, Texas).

3.3.4.1.1 Preparation of FAR1 cDNA

Human FAR1 cDNA sequence was first amplified from pCMV-XL6 by PCR. FAR1 cDNA was then digested as follows: 10 U of *Apa*I, ~2 μg plasmid DNA, and 2 μL proper 10× buffer were supplemented with H_2O to a final volume of 20 μL. The mixture was kept at room temperature for 1 hour and then 10 U of *Nhe*I was added. The DNA was digested at 37 ˚C for another hour. 10 μL of the digestion mixture was resuspended in 2 μL 6× DNA loading dye and separated with a 1% agarose gel containing 1 μg/mL ethidium bromide and 1× TAE (40 mM Tris-Acetate, 1 mM EDTA, pH 8). Electrophoresis was carried out for an hour at 100 volts in 1× TAE buffer. FAR1 DNA fragments shown in the gel were excised and purified by utilizing the QIAquick® Gel Extraction Kit. The procedure followed the manufacturer's direction.

3.3.4.1.2 Ligation of FAR1 cDNA into pFLAG3 and pMyc3

pFLAG3 and pMyc3 vectors were cleaved by *Nhe*I/*Apa*I, separated by agarose gel electrophoresis, excised, and purified by using QIAquick® Gel Extraction Kit. The FAR1 cDNA was ligated into both purified pFLAG and pMyc vectors by using *Nhe*I/*Apa*I restriction sites. This part of work was kindly done by Pam McFie. The ratio of cDNA fragment and vector was 3:1, and the ligation reaction contained 30 fmol of vector, 90 fmol FAR1 cDNA, 10 U T4 ligase and 4 μL 5× ligase buffer with ddH_2O in a final volume of 20 μL. Ligations were performed at 14 ˚C overnight. *E. coli* XL-10 Gold® bacteria were transformed with 2 μL of the ligation mixture. Plasmid DNA was isolated from individual colonies and FAR1 sequence was confirmed by DNA sequencing (PBI-NRC, Saskatoon).

3.4 Mammalian Cell Culture

Human embryonic kidney 293T (HEK-293T) cells and African green monkey kidney cells (COS7) were used for these studies. Both cell lines were cultured at 37 ˚C with 5% CO_2 in 100 mm tissue culture plates containing high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. HEK-293T cells were split the day before transfection at a ratio of 1:5. 20 μg of plasmid DNA was mixed with 430 μL 0.15 M NaCl, and 120 μL 0.1% polyethylenimine. The solution was gently vortexed for 10 seconds and incubated at room temperature for 10 minutes. The transfection mixture was added to 100 mm culture plates. Cells were incubated for 4 hours after which the culture plates were given fresh media.

3.4.1 Oleic Acid Treatment of Cells to Stimulate TG Synthesis

Oleic acid was coupled with BSA to facilitate its uptake by cells. 10% BSA (bovine serum albumin)/7.5 mM oleic acid stock solution was prepared as following: 5 g of fatty acid free BSA was dissolved in 50 mL DMEM and warmed to 56 ˚C. 0.106 g oleic acid was warmed to 56 ˚C and then mixed with the BSA/DMEM solution until clear. The solution was filter sterilized and stored at 4 ˚C. To stimulate TG synthesis, cells were incubated with 0.375 mM oleic acid (diluted with DMEM and 10% FBS) for 24 hours and then harvested.

3.4.2 Cholesterol Treatment of Cells to Stimulate Cholesterol Ester Synthesis

Cholesterol was dissolved in 100% ethanol to generate a cholesterol stock solution (10 mg/mL). For cholesterol treatment, cells in 100 mm plates were incubated with 250 μg cholesterol at a final cholesterol concentration of 25 μg/mL and oleic acid at the concentration of 20 μM for 6 hours. The addition of oleic acid is to stimulate TG synthesis. Cells were then given fresh media and incubated at 37 ˚C for another 24 hours and then harvested.

3.4.3 siRNA Transfection

siRNAs against ACAT1 were chemically synthesized (Invitrogen) and transfected into HEK-293T cells using Lipofectamine™. The sequences of the sense siRNAs were 5-siRNA, 5'-CAGCACACUUGUAGUAGAUUACAUU-3' (designed for targeting 525-549 of ACAT1 coding region mRNA) and 7-siRNA, 5'-CAUGAUCUUCAGAUUGGAGUUCUA-3' (designed for targeting 744-768 of ACAT1 coding region mRNA). For 100 mm plates, 10 mL plating medium was incubated with 2× 1 mL Opti-MEM® I Reduced Serum Medium, 120 pmol siRNA and 20 Lipofectamine™ for 6 hours. Media was replaced and cells were cultured for another 48 hours. Experiments with these siRNAs were compared to untransfected controls. Future studies will use a scrambled sequence.

3.5 Enzyme Activity Assay

3.5.1 *In vitro* DGAT Activity Assay

HEK-293T cells were washed twice with PBS, harvested by scraping and centrifuged for 2 min at 1000 × g. The supernatant was removed, and the cell pellet was resuspended in 400 μL of 50 mM Tris-HCl (pH 7.4) and 250 mM sucrose buffer. To lyse cells, cells were passed through a 30-gauge needle 15 times. The cell debris was pelleted by centrifugation at 600 × g for 5 min, and the supernatant was centrifuged at 100,000 × g for 30 min to pellet cellular membranes. The membrane pellet was resuspended in 400 μL of 50 mM Tris-HCl (pH 7.4) and

250 mM sucrose buffer and the protein concentration was determined as described in section 3.6.1.

DGAT assays were performed as described by Cases *et al.* (1998a). The assay measures the incorporation of [^{14}C]oleoyl CoA into TG under apparent V_{MAX} conditions using exogenous diacylglycerol. 50 μg of membrane proteins were incubated with 20 mM $MgCl_2$, and 100 mM Tris-HCl (pH 7.5) containing 125 μg of BSA, 0.2 mM diacylglycerol and 25 μM [^{14}C]oleoyl CoA (18μCi/μmol) (final volume, 0.2 mL). Reactions were carried out for 10 min at 37 °C and terminated by adding 4 mL chloroform:methanol (2:1 v:v) and 0.8 mL water. After centrifugation at 956 × g for 5 min, the aqueous phase was removed. Lipids in the lower organic phase were dried under a stream of air and separated by thin layer chromatography (TLC) on channeled silica gel G60 TLC plates in hexane:ethyl ether:acetic acid (80:20:1 v:v:v). The bands corresponding to TG were scraped into tubes, and their radioactivity was measured by scintillation counting (Beckman Coulter™, California, U.S.A.).

3.5.2 *In vitro* ACAT Activity Assay

ACAT assays measured the rate of incorporation of [^{14}C]oleoyl CoA into cholesterol esters. Cholesterol was dissolved in acetone, which was allowed to evaporate, and Triton WR-1339 as a 10% (w/v) solution in acetone was added to it at a ratio of 30:1 (w/w). 0.1 M K-phosphate buffer (pH 7.4) was warmed to 60 °C and then added to the cholesterol mixture with constant vortexing, and the organic layer of acetone was dried under a stream of air. The resultant transparent dispersion containing 200 μg/μL of cholesterol was immediately incubated with 120 μg of total membrane proteins. 1 mM glutathione, 0.015 μM BSA and 100 μM [^{14}C]oleoyl CoA were then added (final volume, 0.2 mL). The reaction was initiated by adding [^{14}C]oleoyl CoA, carried out for 15 min at 37°C and stopped by adding 4 mL chloroform:methanol (2:1 v:v) and 0.8 mL water. The incorporation of radioactivity into cholesterol esters was determined as described for DGAT assays.

3.6 Immunoblot Analysis

3.6.1 Protein Determination

The principle of the protein assay was to compare the absorbance maximum for Coomassie blue shifting from 465 nm to 595 nm by binding to protein. In a protein assay, 20 μL of proteins was added to 100 μL reagent A, and then mixed with 800 μL reagent B (Bio-Rad). Samples were incubated at room temperature for 15 min, and then read at $O.D._{595}$ using a spectrophotometer (SmartSpec™, Bio-Rad). A protein standard curve was prepared by using protein standards ranging from 0.2 mg/mL to 1.5 mg/mL. Next, the concentrations of protein standards were plotted against the corresponding absorbance resulting in a standard curve. The concentration of the sample protein was thus determined.

3.6.2 Preparation of Solubilized Membrane Proteins

Transfected HEK-293T cells or COS7 cells were collected by centrifugation (86 × g, 2 min), solubilized with 400 μL 0.5% CHAPS in PBS in the presence of protease inhibitors and passed through a 30-gauge needle 15 times. Cell debris was removed by centrifugation at 16,873 × g for 10 min at 4 °C. The resulting supernatant contained solubilized membrane proteins. Protein content was determined as described in last section.

3.6.3 SDS Polyacrylamide Gel Electrophoresis

Protein samples were prepared by diluting 40 μg of protein to 1 μg/μL in 5× SDS-PAGE sample buffer (250 mM Tris, pH 6.8, 10% SDS, 50% glycerol, 0.5% bromophenol blue, and 25% β-mercaptoethanol). When immunoblotting DGAT2, protein samples were heated to 100°C for 5 min. When immunoblotting DGAT1, ACAT1 and FAR1, protein samples were incubated at 37°C for 15 min to prevent aggregation. Denatured proteins were separated on a 10% SDS polyacrylamide gel consisting of 30% N,N'-methylene-bis-acrylamide (29:1), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate (APS), and 0.0004% N,N,N',N'-tetramethylethanediamine (TEMED) with a 5% stacking gel consisting acrylamide, 130 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS and 0.001% TEMED. PageRuler™ prestained protein

ladder Plus was used to estimate protein size. Electrophoresis was carried out at 160 volts in running buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS.

After separation by electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane. The transferring process was carried out at 300 mA for 80-90 min in transfer buffer containing 62.5 mM boric acid, 5 N NaOH, pH 8. The membrane was blocked in 5% non-fat milk/ PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, 0.5% TWEEN® 20, pH 7.4) for 1 h at room temperature with constant shaking. Blocking buffer was then changed with fresh buffer containing the appropriate primary antibody. The membrane was incubated at room temperature for another hour and then washed five times with fresh PBS-T for 5 min each. Fresh PBS-T buffer containing secondary antibody linked to horseradish peroxidase was applied to the membrane for next hour. The membrane was then washed four times with PBS-T and one time with PBS for 5 min each. The protein-antibody complex on the membrane was next incubated with commercial chemiluminescent reagents for 5 min (SuperSignal® West Pico Chemiluminescent Substrate) or home-made chemiluminescent reagents (250 mM luminol, 40 mM coumaric acid, 1 M Tris-HCl pH 8.5 and 0.03% hydrogen peroxide [final volume: 20 mL]) for 1 min and then exposed to film. The list of antibodies is in Table 3.2.

3.7 Lipid Analysis

3.7.1 Lipid Composition by Charring of TLC plates

Cell lysates containing 750 μg protein were diluted to a final volume of 1 mL with water. 4 mL chloroform:methanol (2:1 v:v) was added, vortexed for 30 sec and the mixture was then centrifuged at 956 ×g for 5 min. The upper aqueous phase was removed and the organic phase was dried under a stream of air. Samples were resuspended in 60 μL of chloroform:methanol (2:1 v:v) and applied to a TLC plate. Lipids were separating using the solvent system hexane:ethyl ether:acetic acid (80:20:1 v:v:v). The TLC plate was then air-dried, placed in a solution containing 10% cupric sulphate (w/v) and 8% phosphoric acid for 20 sec. The TLC plate was heated in an oven at 180°C for ~7 min until lipids were visible. Lipid levels were quantified by densitometry and were linear within the range of our assay. Densities were determined using ImageJ software (National Institutes of Health).

3.7.2 Triacylglycerol Synthesis in Intact Cells

Triacylglycerol synthesis in intact cells was determined by measuring the incorporation of [^{3}H]glycerol into TG. HEK-293T cells were incubated with 10 μCi [^{3}H]glycerol and 0.5 mM oleic acid in growth medium for 6 h. Lipids were extracted from cell lysates and separated as described in Section 3.7.1. Bands corresponding to TG were scraped from the TLC plate and the incorporation of radioactivity into [^{3}H]TG was determined by liquid scintillation counting.

3.8 Immunoprecipitation of DGAT1

HEK-293T cells were lysed with 0.5% CHAPS in PBS by passing cells through a 30-gauge needle 15 times. The cell debris was pelleted by centrifugation at 600 × g for 5 min, and the supernatant was centrifuged at 100,000 × g for 30 min at 4°C to pellet cellular membranes. Resultant cell pellets were resuspended in 400 μL of 0.5% CHAPS in PBS and pre-cleared by incubation with 50 μL Protein A immobilized on Sepharose beads for 1 h at 4°C while rotating to minimize non-specific binding. Protein A Sepharose beads were prepared according to manufacturer's direction. Pre-cleared cell lysates was incubated with monoclonal Anti-FLAG M2 agarose beads (Sigma-Aldrich) for 2 h at 4°C while rotating, and washed with 0.5% CHAPS in PBS for five times. Each washing used 1 mL buffer for 5 min, and proteins conjugated to antibodies on beads were pelleted by centrifugation for 1 min at 16,873 × g. Samples were eluted by 3 × FLAG® Peptide (Sigma-Aldrich), 3 μL of which were incubated with proteins derived from one plate. The immunoprecipitates were stored at -80°C, if not used immediately.

3.9 Mass Spectrometry and Identification of DGAT1 Interacting Proteins

Immunoprecipitates were subjected to SDS-PAGE and stained with Coomassie blue to visualize immunoprecipitated proteins. Bands corresponding to DGAT1 as well as the compartment bands in untransfected cells were excised and sent for analysis by tandem mass spectrometry (service provided by Plant Biotechnology Institute, Saskatoon, SK). The obtained data were searched against both NCBInr 04012010 and Swiss Prot 57.15 databases (Taxonomy:

Homo sapiens). Proteins were predicted by the Mascot search engine, and the result was the probability indicating the match of the spectrum to a protein.

4. RESULTS

4.1 Identification of DGAT1-Interacting Proteins

Triacylglycerols are synthesized by DGAT enzymes at the ER and stored in cytosolic lipid droplets. Cholesterol esters are synthesized at the ER by ACAT and stored in lipid droplets as well. However, the formation of lipid droplets is a poorly understood process. It is also unclear how TG and cholesterol esters produced by DGAT enzymes and ACAT enzymes at the ER are incorporated into lipid droplets. Since almost all cellular processes require protein-protein interactions, DGAT1 may also have protein partners that facilitate TG synthesis and its storage into lipid droplets. To identify DGAT1-interacting proteins, we used co-immunoprecipitation in conjunction with mass spectrometry.

Co-immunoprecipitation is a technique routinely utilized to identify protein-protein interactions in an unbiased manner. Proteins are precipitated out of the solution using a specific antibody that recognizes a bait protein. For the following experiments, DGAT1 containing a FLAG epitope at its N-terminus was expressed in HEK-293T cells and was immunoprecipitated using an anti-FLAG antibody. Proteins bound to DGAT1 should be present in the immunoprecipitates and their identities can be determined by mass spectrometry. One of the limitations of co-immunoprecipitation is that weakly interacting proteins can dissociate during experimental manipulations. The other limitation of this approach is that complex protein mixtures make it difficult to conclude that two interacting proteins bind each other directly (Masters, 2004; Anderson, 1998).

4.1.1 Immunoprecipitation of DGAT1

HEK-293T cells were chosen to overexpress DGAT1. FLAG-tagged murine DGAT1 (FL-DGAT1) cDNA was transiently transfected into HEK-293T cells. DGAT1 is approximately 55 kDa, and in SDS-PAGE it usually migrates near 50 kDa. The expression of DGAT1 was confirmed by immunoblotting with anti-FLAG (Figure 4.1A). Protein extracts from

untransfected HEK-293T cells and HEK-293T cells expressing FL-DGAT1 were then solubilized with 0.5% CHAPS and pre-cleared by incubation with protein A Sepharose for 30 min on a rotating platform to minimize non-specific binding. DGAT1 was immunoprecipitated with anti-FLAG and detected with anti-FLAG antibodies by immunoblot analysis (Figure 4.1B). When the immunoprecipitates were stained with Coomassie Blue, several protein bands were detected in the DGAT1 immunoprecipitates that were absent in the immunoprecipitates from untransfected cells (Figure 4.1C). These proteins were excised and identified by mass spectrometry.

4.1.2 Protein Identification by Mass Spectrometry

Mass spectrometry is used to analyze the mass-to-charge ratio of unknown charged particles or peptides. Immunoprecipitates were separated by SDS-PAGE and stained with Coomassie Blue. Protein bands visualized in DGAT1 immunoprecipitates were designated P6b, P7b and P8b and their counterparts in untransfected immunoprecipitates were designated P6a, P7a and P8a (Figure 4.1C). These bands in the gels were excised and sent to the mass spectrometry core facility at NRC-PBI (National Research Council Plant Biotechnology Institute) for identification of candidate interacting proteins of DGAT1.

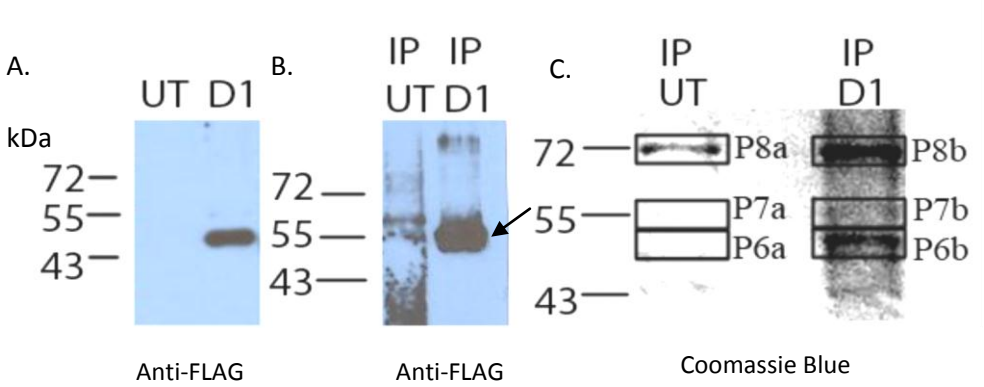


Figure 4.1. Immunoprecipitation of DGAT1 expressed in HEK-293T Cells. (A) By immunoblot analysis, cell lysates from HEK-293T transfected with FL-DGAT1 and untransfected cells were immunoblotted with anti-FLAG. (B) Immunoprecipitates from HEK-293T cells expressing FL-DGAT1 and untransfected cells were immunoblotted with anti-FLAG. DGAT1 is indicated by the arrow. (C) Same IP samples of DGAT1 and untransfected cells were stained with Coomassie Blue. Bands labeled as P6b, P7b and P8b in D1 samples and bands labeled as P6a, P7a and P8a in UT samples were excised and sent for Mass spectrometry. D1, DGAT1. UT, untransfected cells. IP, immunoprecipitation.

Proteins were identified by comparison to databases of NCBInr 04012010 and SwissProt 57.15, and those both present in immunoprecipitates from DGAT1 and untransfected cells were considered to be a result of non-specific binding. Candidate binding proteins are listed in Table 4.1.

Table 4.1: List of Candidate Binding Proteins from Mass Spectrometry

	Abbr.	Full Name	Function Description/ Peptides
P6	ACAT1	Acyl-CoA:cholesterol acyltransferase	Cholesterol ester synthesis (Lee *et al*., 1998). Peptides matched: "KESLETPSNGRI", "KEVGSHFDDFVTNLIEKS".
	PTSS2	Phosphatidylserine synthase 2	PS synthesis (Bergo *et al*., 2002). Peptide matched: "RDAGGPRPESPVPAGRA".
	APMAP	Adipocyte plasma membrane-associated protein	A transcript required for adipogenesis (Bogner-Strauss *et al*., 2010). Peptides matched: "KLLLSSETPIEGKN", "KGLFE-VNPWKR".
P7	SPTC1	Serine palmitoyltransferase 1	Sphingolipid synthesis (Hanada, 2003). Peptide matched: "RVVVTVEQTEEELER-A", "KTEEAIIYSYGFATIASAIPAYSKR".
	LPCAT1	Lysophosphatidylcholine acyltransferase 1	Phosphatidylcholine synthesis (Nakanishi *et al*., 2006) Peptide matched: "KTALGVAE-LTVTDLFRA".
	FAR1	Fatty acyl-CoA reductase 1	Wax biosynthesis (Cheng and Russell, 2004a; Cheng and Russell, 2004b). Peptide matched: "KAPAFLYDIYLRM".
	DHCR24	24-dehydrocholesterol reductase	Cholesterol biosynthesis (Waterham *et al*., 2001). Peptide matched: "REGLEYIPLRH".

4.2 Interaction of DGAT1 and ACAT1

4.2.1 DGAT1 Interacts With ACAT1

We first chose to investigate the role of ACAT1 as a DGAT1 interacting protein since ACAT1 also uses fatty acyl-CoA as a substrate and ACAT1 is related to DGAT1. This common feature of DGAT1 and ACAT1 might enable them to accept fatty acyl-CoA from possibly the same acyl donor carrier protein and thus DGAT1 and ACAT1 could interact. Since DGAT2 catalyzes TG synthesis, we were also interested if ACAT1 interacted with DGAT2. FL-DGAT1 and FLAG-tagged DGAT2 (FL-DGAT2) were transfected into HEK-293T cells. We chose HEK-293T cells as the have the fundamental pathway for TG biosynthesis present. FL-DGAT1 and FL-DGAT2 were then immunoprecipitated using anti-FLAG coupled to agarose. Immunoprecipitates were immunoblotted with anti-FLAG to confirm the presence of FL-DGAT1 and FL-DGAT2 (Figure 4.2A). To verify whether ACAT1 could be co-immunoprecipitated with DGAT1 and DGAT2, we probed FL-DGAT1 and FL-DGAT2 immunoprecipitates with an ACAT1 antibody. ACAT1 (~50kDa) was present in FL-DGAT1 immunoprecipitates, but absent in FL-DGAT2 and untransfected immunoprecipitates (Figure 4.2B). This data suggested that ACAT1 interacted with DGAT1, but not DGAT2. To verify that the visualized ACAT1 band was not caused by a non-specific reaction between the secondary antibody and ACAT1, the immunoprecipitates were blotted with secondary antibody only (Figure 4.2C). The ACAT1 band was not detected in the absence of ACAT1 antibody. Combined with the result of mass spectrometry, this experiment confirmed that ACAT1 could interact with DGAT1, but not DGAT2.

4.2.2 Effect of ACAT1 Knockdown on DGAT Activity and TG Synthesis

We next determined whether the interaction of DGAT1 and ACAT1 could influence DGAT1 function and TG synthesis. Since DGAT1 and ACAT1 share a common substrate, fatty acyl-CoA, we hypothesized that ACAT1 competed with DGAT1 for this substrate and the knockdown of ACAT1 could increase TG synthesis. To address this question, we suppressed

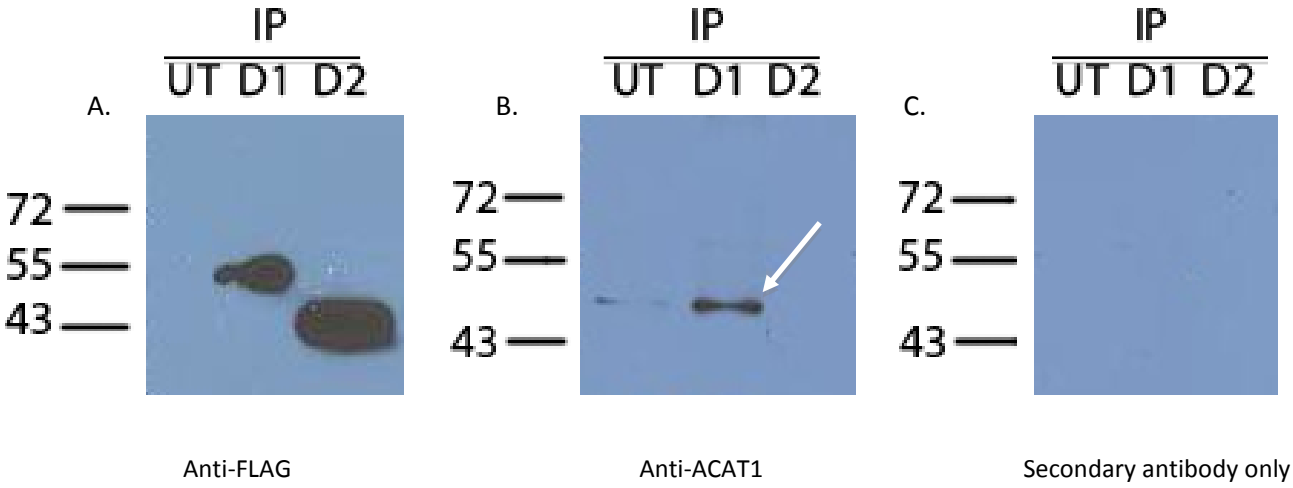


Figure 4.2. DGAT1 and ACAT1 interact with each other. (A) Immunoprecipitates from HEK-293T cells expressing FL-DGAT1, FL-DGAT2 and untransfected cells were analyzed by immunoblot analysis using anti-FLAG. (B) Immunoprecipitates from HEK-293T cells expressing FL-DGAT1, FL-DGAT2 and untransfected cells were analyzed by immunoblot analysis using anti-ACAT1. The ACAT1 band was indicated with a white arrow. (C) Same IP samples from the last experiment were immunoblotted with secondary antibody only. UT, untransfected cells. IP, immunoprecipitates.

ACAT1 expression with two different small interfering RNA (siRNA) oligonucleotides, 5-siRNA and 7-siRNA, and comparing siRNA-transfected cells to untransfected cells. Immunoblot analysis showed that ACAT1 was successfully knocked down by either 5-siRNA or 7-siRNA (Figure 4.3). We next examined the consequences of ACAT1 knockdown on DGAT activity and TG synthesis. Unexpectedly, *in vitro* DGAT activity was decreased when ACAT1 was knocked down with 5-siRNA (Figure 4.4). No consistent decrease in DGAT activity was observed for 7-siRNA. Therefore, the interaction of DGAT1 and ACAT1 may stabilize DGAT1, and the disruption of it could increase DGAT1 degradation leading to decreased DGAT activity. We also examined the effect of ACAT1 knockdown on TG synthesis in intact HEK-293T cells. The knockdown of ACAT1 was verified by immunoblot analysis (Figure 4.5). Lipids in HEK-293T cells were extracted, separated by TLC and visualized by charring. Oleic acid can stimulate TG synthesis in the cell. If ACAT1 knockdown affected TG synthesis, ACAT1-knockdown cells would not response to oleic acid supplementation as much as wild-type cells do and TG levels in

ACAT1-knockdown cells would be much lower in the presence of oleic acid compared to wild-type cells.

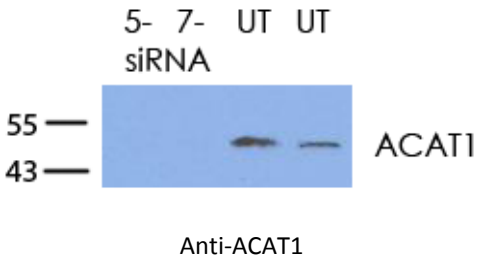


Figure 4.3. ACAT1 knockdown using siRNAs. Immunoblot analysis for ACAT1 expression in cell lysates from HEK-293T cells transfected with ACAT1 siRNAs and untransfected cells. 5-, 7-siRNA, different ACAT1-specific siRNA oligonucleotides. UT, untransfected cells.

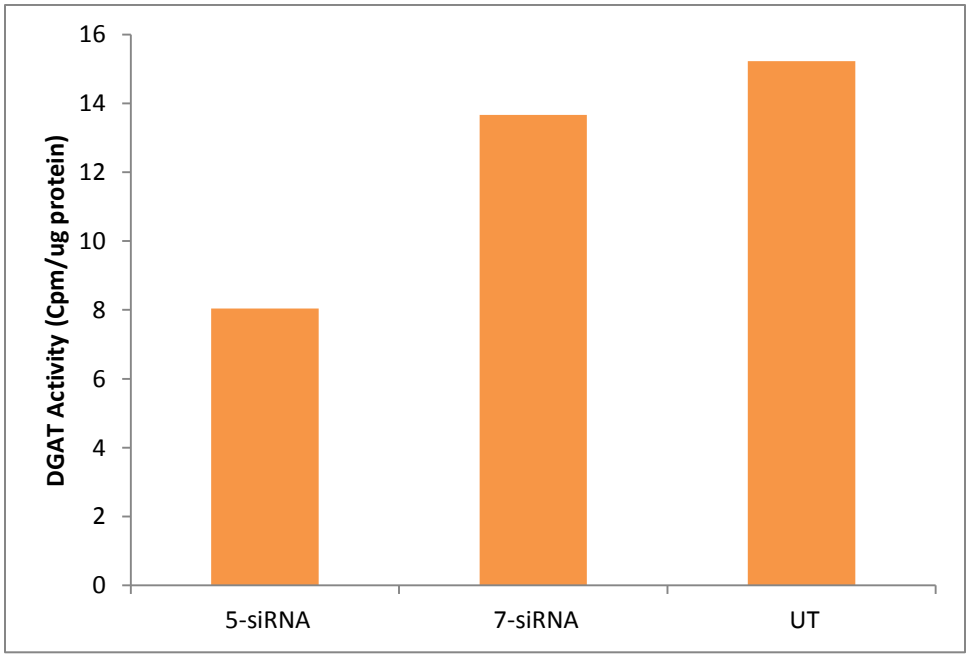


Figure 4.4. The effect of ACAT1 knockdown on DGAT activity. DGAT activity analysis in cell lysates from HEK-293T cells transfected with ACAT1 siRNAs and untransfected cells. 5-, 7-siRNA, different ACAT1-specific siRNA oligonucleotides. UT, untransfected cells. n=2 samples of each group. The experiment was repeated with similar results.

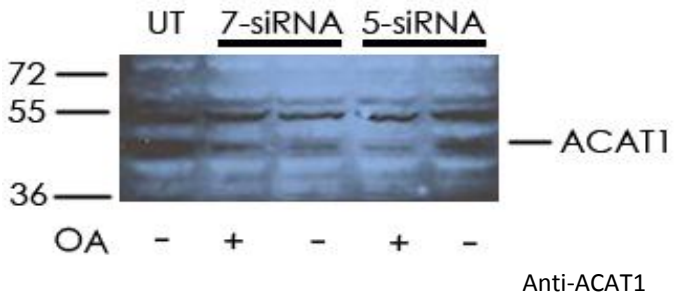


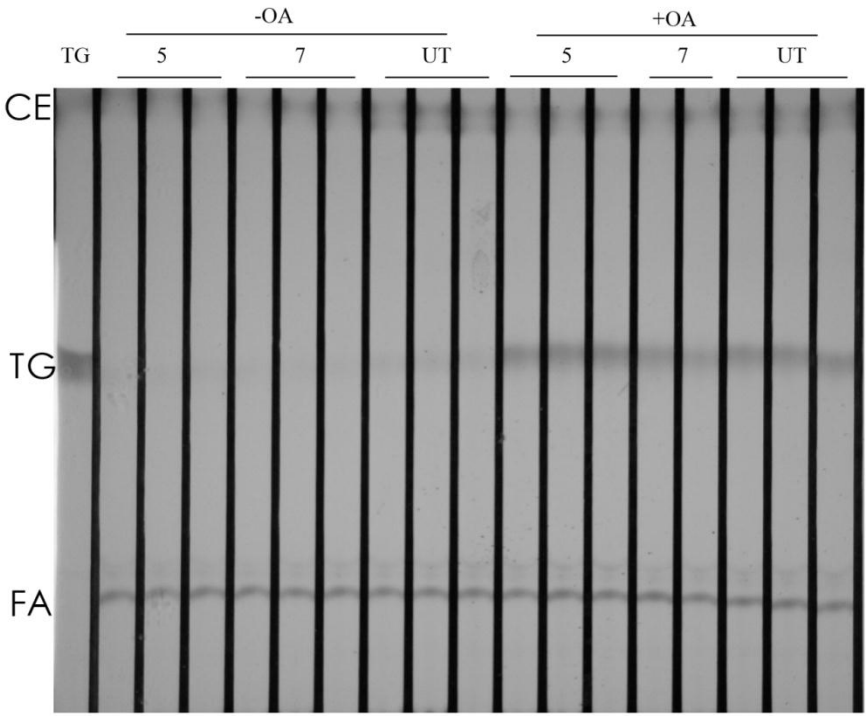
Figure 4.5. The silencing of ACAT1 in +/- oleic acid conditions. Immunoblot analysis for ACAT1 expression in cell lysates from untransfected cells and HEK-293T cells transfected with ACAT1 siRNAs in the presence or absence of 0.375mM oleic acid. OA, oleic acid. "+" and "-", in presence or absence of oleic acid.

As shown, cholesterol ester levels decreased due to ACAT1 knockdown in both the presence and absence of added oleic acid (Figure 4.6A). In the absence of oleic acid, TG levels declined in all siRNA-transfected samples. In the presence of oleic acid, neither 7-siRNA nor 5-siRNA could significantly decrease TG levels. This result was probably due to endogenous DGAT2 using oleic acid for TG synthesis. The fact that ACAT1 knockdown decreased TG level in the absence of oleic acid suggested that TG synthesis was dependent on the interaction of DGAT1 and ACAT1.

4.2.3 DGAT1 Does Not Have ACAT Activity and ACAT1 Does Not Have DGAT Activity

Previous studies have shown that DGAT1 does not have ACAT activity and ACAT1 does not have DGAT activity (Cases *et al*., 1998a; Yen *et al*., 2005). Due to the finding that ACAT1 knockdown decreased not only cholesterol ester levels but also TG levels, it was necessary to confirm the former conclusions in our studies. HEK-293T cells were transfected with either DGAT1 or ACAT1. In DGAT activity assays, DGAT1-transfected cells showed increased DGAT activity, while ACAT1-transfected cells did not (Figure 4.7). ACAT activity assays showed that ACAT1 overexpression increased ACAT activity, whereas DGAT1 overexpression did not.

A.



B.

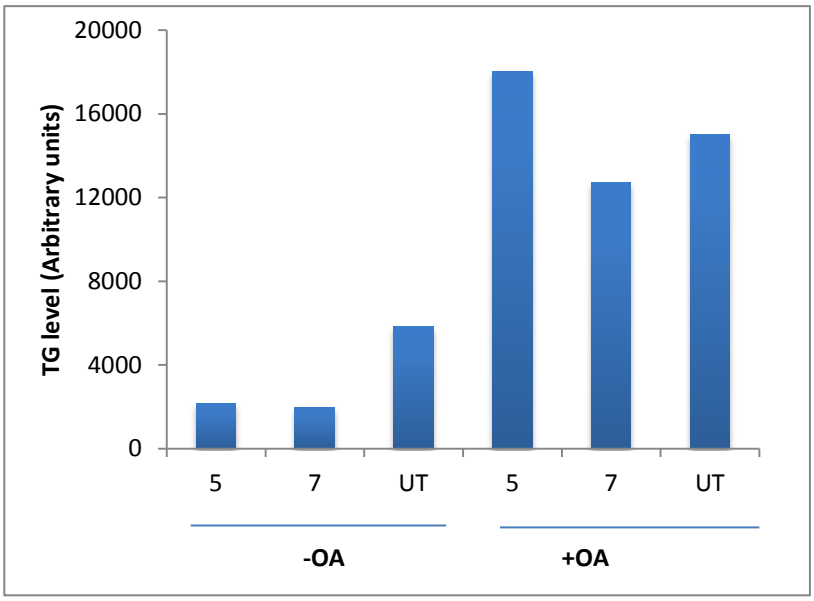


Figure 4.6. Lipid profile of HEK-293T cells transfected with ACAT1-specific siRNA with +/- oleic acid. (A) Total lipids were extracted from untransfected cells and HEK-293T cells transfected with siRNAs in the presence or absence of 0.375mM oleic acid. Lipid extracts were analyzed by thin layer chromatography. (B) TG was quantified by densitometry using ImageJ and compared in chart. UT, untransfected cells. OA, oleic acid. n=3 samples for each group. The experiment was repeated with similar results.

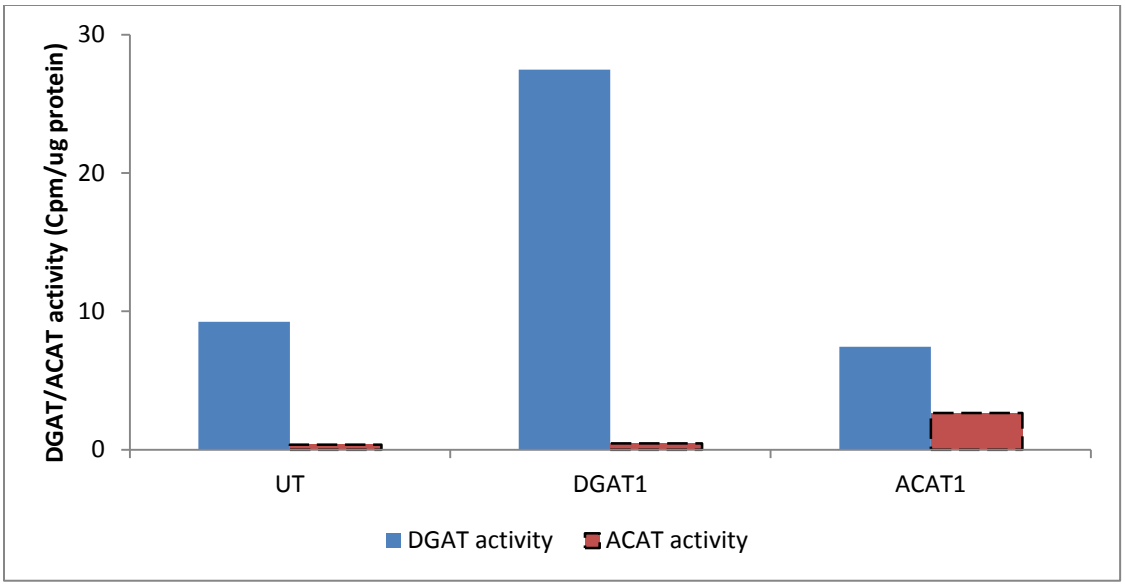


Figure 4.7. Acyltransferase activities of DGAT1 and ACAT1. DGAT and ACAT activity analysis in cell lysates from HEK-293T cells transfected with DGAT1 or ACAT1 and untransfected cells. n=3 samples for each group. The experiment was repeated with similar results.

4.2.4 Effect of ACAT1 and DGAT1 Overexpression on TG and Cholesterol Ester Synthesis

If silencing ACAT1 could decrease TG levels in the absence of fatty acids, we hypothesized that ACAT1 overexpression would increase TG levels. To test this hypothesis, HEK-293T cells were transfected with the expression vector of ACAT1 and supplemented with oleic acid or cholesterol. The purpose of cholesterol treatment is that cholesterol activates ACAT1 and supplies substrates for cholesterol ester synthesis (Zhang *et al*., 2003). If the interaction between DGAT1 and ACAT1 could influence the synthesis of their respective products, we hypothesized that cholesterol-stimulating ACAT1 overexpression increased TG and cholesterol ester synthesis. ACAT1 overexpression was confirmed using anti-ACAT1 (Figure 4.8A). In the presence and absence of oleic acid or cholesterol, ectopic ACAT1 expression increased both cholesterol ester and TG production, especially with the addition of cholesterol (Figure 4.8B and 4.8C). The reason why ACAT1 could increase TG synthesis was probably that ACAT1 overexpression up-regulated DGAT activity or the DGAT1 protein level that resulted in more functional ACAT1-DGAT1 complex forming, and thereby producing TG. This finding fur-

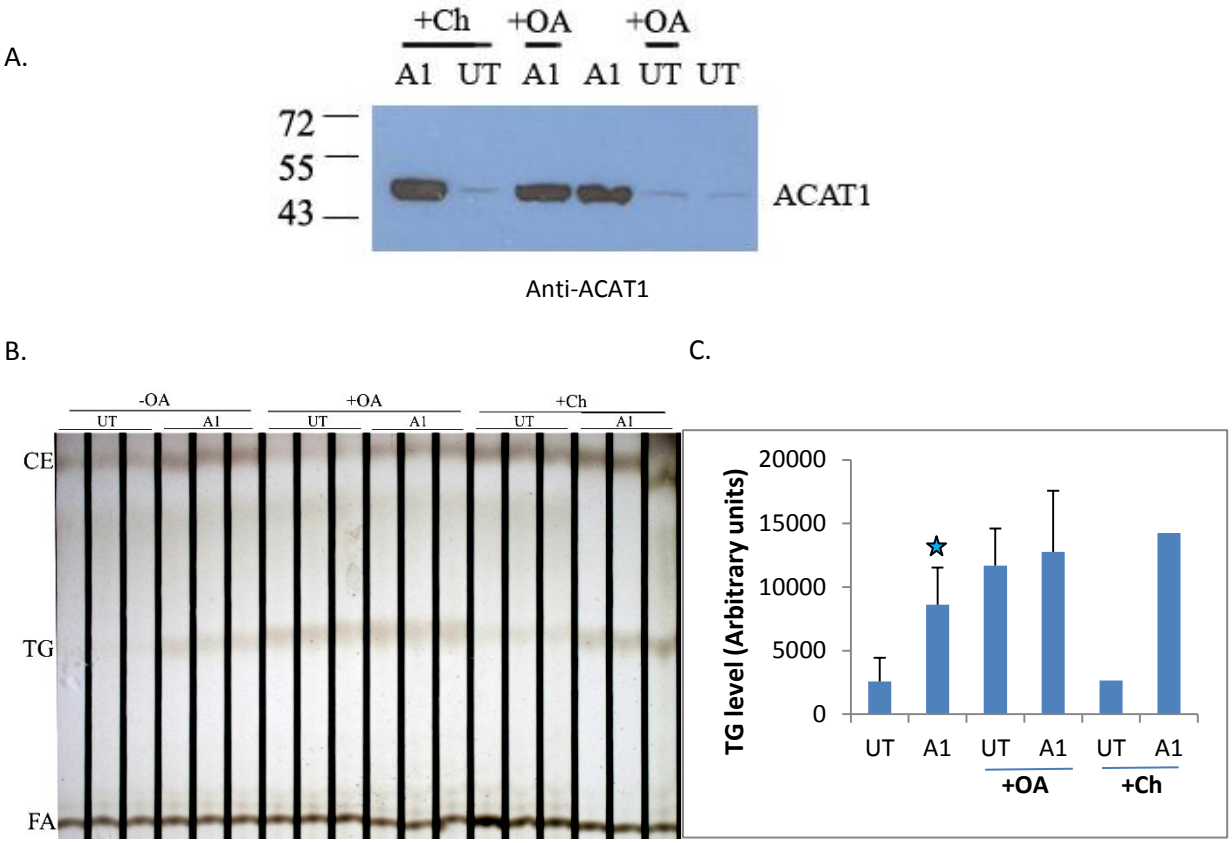


Figure 4.8. Lipid profile of HEK-293T cells overexpressing ACAT1 in +/- oleic acid and +/- cholesterol conditions. (A) Immunoblot analysis for ACAT1 expression in cell lysates from untransfected cells and HEK-293T cells transfected with ACAT1 in the presence or absence of 0.375mM oleic acid and 25 µg/mL cholesterol. (B) Total lipids were extracted from untransfected cells and HEK-293T cells transfected with ACAT1 in the presence or absence of oleic acid and cholesterol. Lipid extracts were analyzed by thin layer chromatography. (C) TG was quantified by densitometry using ImageJ. *, $P<0.05$, versus UT. OA, oleic acid. Ch, cholesterol. UT, untransfected cells. Error bars stand for standard error of the means. n=3 samples for each group. The experiment was repeated with similar results.

ther supported a cooperative the hypothesis of a relationship between ACAT1 and DGAT1.

To better understand the results of how ACAT1 could increase TG synthesis under +/- oleic acid and cholesterol, we individually transfected ACAT1 or FL-DGAT1 in +/- cholesterol conditions and tested their effects on lipid synthesis. FL-DGAT1 and ACAT1 expression was confirmed by immunoblot analysis with anti-FLAG or anti-ACAT1, respectively (Figure 4.9A).

Both overexpression of ACAT1 and FL-DGAT1 could increase cholesterol ester level in +/- cholesterol (Figure 4.9B and 4.9C). Furthermore, in +/- cholesterol, ACAT1-transfected cells produced more TGs than the control.

Our explanation for these findings was that DGAT1 and ACAT1 assisted each other to synthesize TG and cholesterol esters, respectively. Since we have confirmed that DGAT1 and ACAT1 interact with each other, we propose that DGAT1 and ACAT1 interact as part of a lipid-

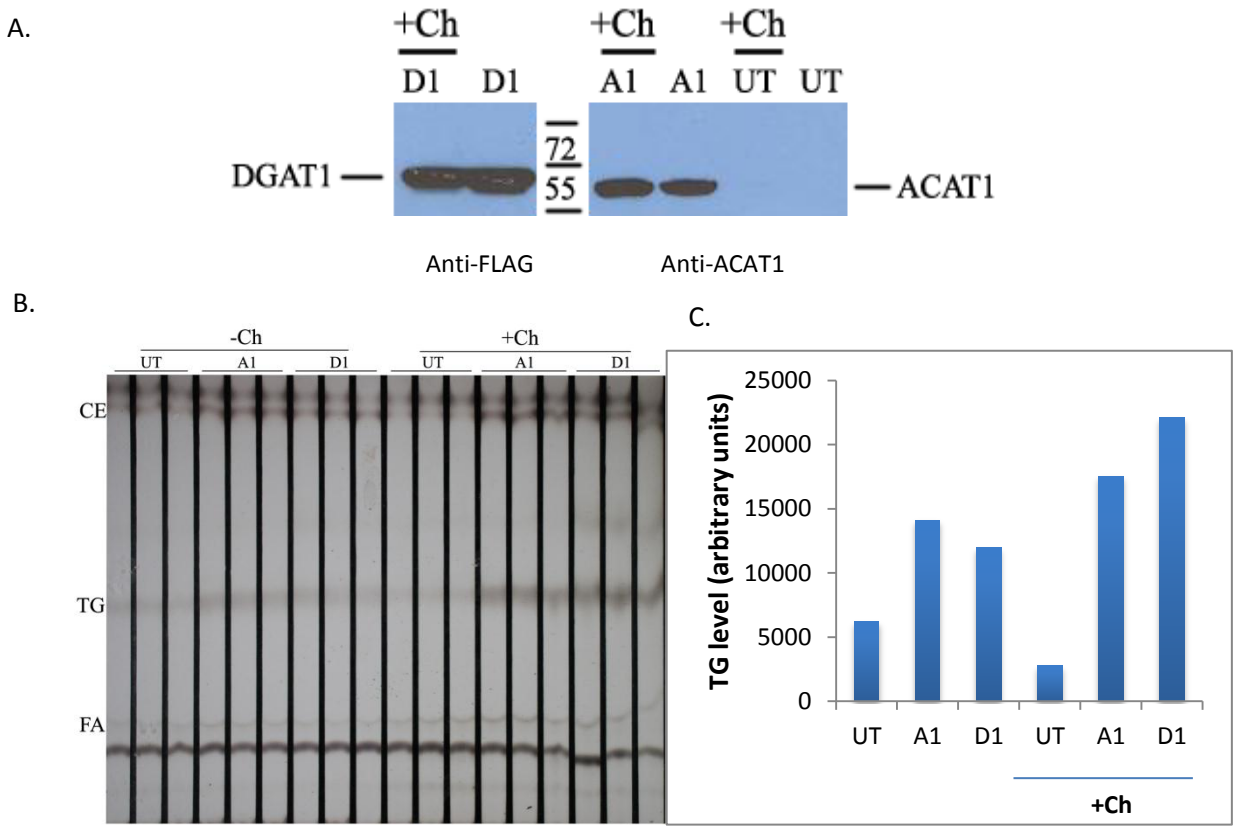


Figure 4.9. Lipid analysis on HEK-293T cells overexpressing DGAT1 and ACAT1 in +/- cholesterol conditions. (A) Immunoblot analysis for FL-DGAT1 and ACAT1 expression in cell lysates from untransfected cells and HEK-293T cells transfected with FL-DGAT1 and ACAT1 in the presence or absence of 25 µg/mL cholesterol. (B) Total lipids were extracted from untransfected cells and HEK-293T cells transfected with FL-DGAT1 and ACAT1 in the presence or absence of cholesterol. Lipid extracts were analyzed by thin layer chromatography. (C) TG was quantified by densitometry using ImageJ. A1, ACAT1. D1, FL-DGAT1. UT, untransfected cells. -Ch, normal condition without cholesterol. +Ch, cholesterol treatment. n=3 samples for each group. The experiment was repeated with similar results.

synthesizing complex, or DGAT1-ACAT1 protein complex, producing both TG and cholesterol esters. Either increasing the amount of DGAT1 or ACAT1 protein could lead to more DGAT1-ACAT1 protein complex formation, and thus increase the product synthesis of both of them. This was why ACAT1 transfection increased TG synthesis.

4.2.5 Identification of the Domain of DGAT1 that Interacts with ACAT1

We next tried to identify which region of DGAT1 was responsible for interacting with ACAT1. The approach was to determine if ACAT1 could be co-immunoprecipitated with various DGAT1 mutants that had been generated from a previous study (McFie *et al*., 2010). DGAT1 and all DGAT1 mutants (Δ1-37, Δ38-84 and Δ1-85) had a FLAG tag in their N-terminus (Figure 4.10A). FL-DGAT1 and the FL-DGAT1 mutants, Δ1-37, Δ38-84 and Δ1-85, were expressed in HEK-293T cells and immunoprecipitated with anti-FLAG. The immunoprecipitates were then immunoblotted with anti-FLAG and anti-ACAT1 antibodies. Immunoblot analysis with anti-FLAG confirmed that FL-DGAT1 and the three mutants were successfully immunoprecipitated (Figure 4.10B). However, ACAT1 was also detected in the immunoprecipitates of all three FL-DGAT1 mutants, indicating that DGAT1 did not interact with ACAT via its N-terminal domain (Figure 4.10C).

4.3 DGAT1 Does Not Interact with FAR1

We next studied FAR1, another potential DGAT1 interacting protein identified by mass spectrometry. HEK-293T cells were transfected with FL-DGAT1, Myc-FAR1 and FL-DGAT1 and Myc-FAR1 together, respectively. The expression of FL-DGAT1 and Myc-FAR1 was confirmed by immunoblotting cell lysates with anti-FLAG and anti-Myc (Figure 4.11A and B, lanes of cell lysates). Solubilized cell lysates containing FL-DGAT1 were incubated with anti-FLAG and FL-DGAT1 was immunoprecipitated and immunoblotted by anti-FLAG (Figure 4.11A, lanes of IP).

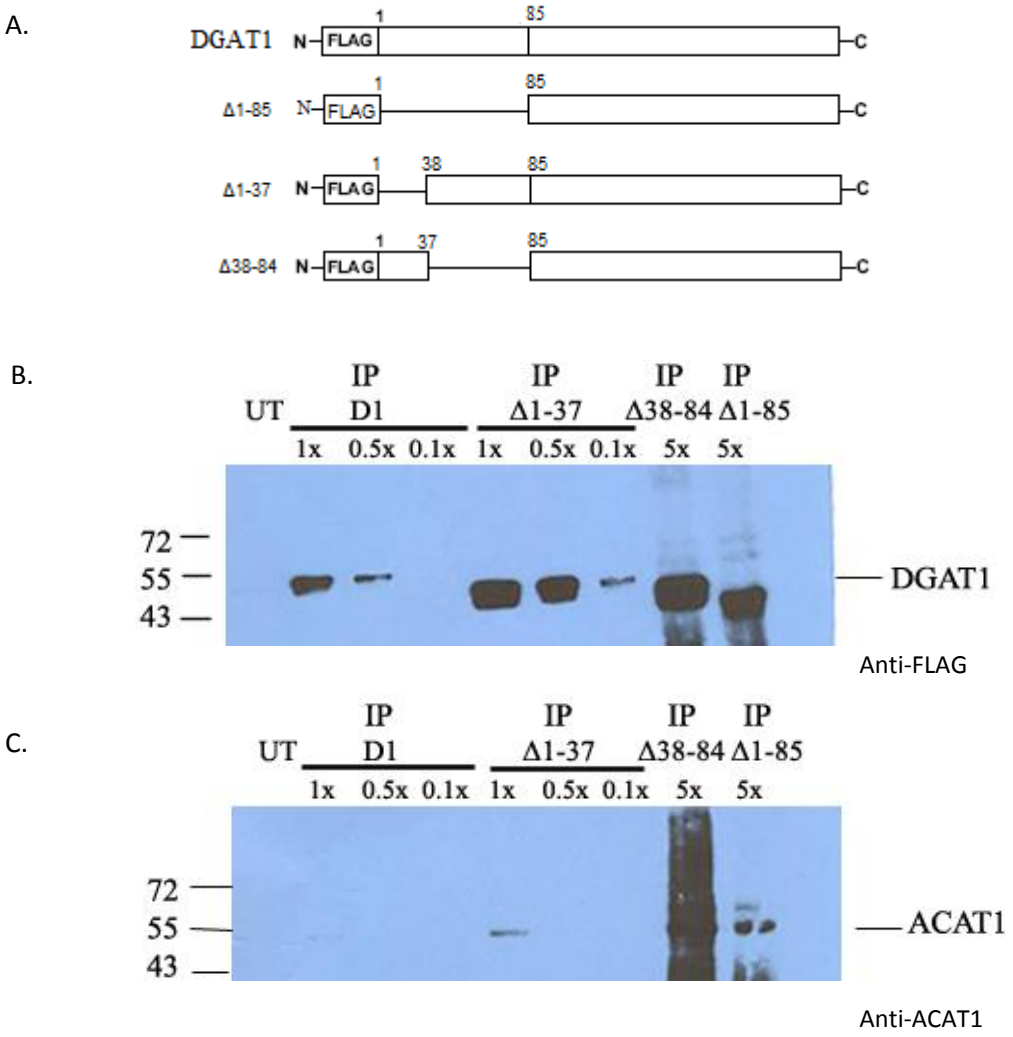


Figure 4.10. Identification of the region of DGAT1 interacting with ACAT1. (A) A map of DGAT1 mutant proteins. (B) Full-length or deletion mutants of FL-DGAT1 expressed in HEK-293T cells were immunoprecipitated and analyzed by immunoblotting using anti-FLAG. (C) Endogenous ACAT1 was immunoblotted by using anti-ACAT1 in FL-DGAT1 and FL-DGAT1 mutants' immunoprecipitates. 5X, sample loaded with 7.5µl IP elution. 1X, 1.5µl. 0.5X, 0.75µl. 0.1X, 0.15µl. UT, untransfected cells. IP, immunoprecipitation.

However, for co-immunoprecipitation, Myc-FAR1 was shown present in immunoprecipitates from cells expressing only Myc-FAR1 as well as immunoprecipitates from cells co-expressing FL-DGAT1 and Myc-FAR1 (Figure 4.11B, lanes of IP). This result suggested that Myc-FAR1 was pulled down non-specifically. If DGAT1 interacted with FAR1, then Myc-FAR1 should be pulled down when co-expressed with FL-DGAT1. However, the

result showed that the Myc-FAR1 level from Figure 4.11B did not increase significantly. Therefore, it appears that Myc-FAR1 was pulled down due to non-specific binding of FAR1 with anti-FLAG.

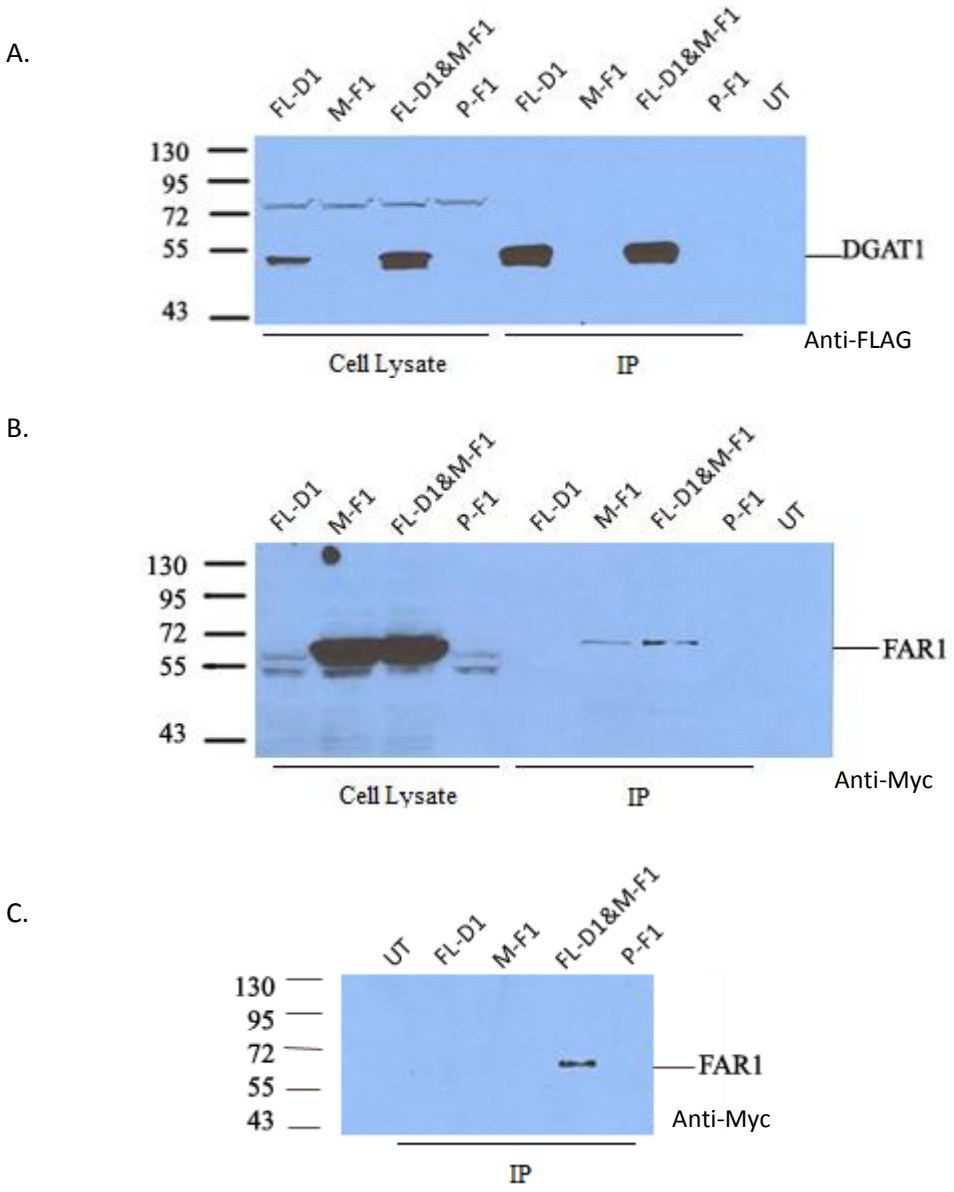


Figure 4.11. DGAT1 and FAR1 do not interact. (A) Immunoblot analysis of DGAT1 expression and immunoprecipitation from HEK-293T cells transfected with FL-DGAT1, Myc-FAR1, FL-DGAT1 and Myc-FAR1 and P-FAR1. (B) Immunoblot analysis of FAR1 expression and immunoprecipitation was prepared in the same way as the last experiment. (C) The repeat of the experiment shown in Fig. 4.11B. UT, untransfected cells. FL-D1, FL-DGAT1. M-F1, Myc-FAR1. FL-D1&M-F1, FL-DGAT1 and Myc-FAR1 co-transfection. P-F1, FAR1 without any epitope. UT, untransfected cells. IP, immunoprecipitation.

If DGAT1 and FAR1 indeed interacted, the result of co-immunoprecipitation shown should be like Figure 4.11C, in which Myc-FAR1 was present in co-transfection immunoprecipitates and absent in single-transfection immunoprecipitates. But the result in Figure 4.11C was not repeatable. Since DGAT1 is localized to the ER and FAR1 is present in peroxisome, it is unlikely that DGAT1 and FAR1 interact.

4.4 Interaction of DGAT1 and DGAT2

To determine if DGAT1 interacted with DGAT2, I co-expressed Myc-DGAT1 and FL-DGAT2 in COS7 cells (African green monkey kidney cells). The reason that we switched the cell line from HEK-293T to COS7 was due to the promoter of FL-DGAT2, pEF-1α, was not active in HEK-293T cells. The co-expression of both FL-DGAT2 (predicted size: ~45 kDa, actual size: ~40 kDa) and Myc-DGAT1 was confirmed by immunoblot analysis with anti-FLAG and anti-Myc antibodies (Figure 4.12A and B). FL-DGAT2 was immunoprecipitated with anti-FLAG, which was confirmed by immunoblot analysis (Figure 4.12C). Immunoprecipitates were then immunoblotted with anti-Myc (Figure 4.12D). Surprisingly, Myc-DGAT1 was present in the anti-FLAG immunoprecipitates, and suggested that DGAT1 could interact with DGAT2.

4.4.1 Effect of DGAT1 and DGAT2 Interaction on Lipid Synthesis

Based on our finding that DGAT1 and DGAT2 interacted, we hypothesized that DGAT1 and DGAT2's interaction could facilitate lipid synthesis. To test this, HEK-293T cells were used for the co-transfection of FL-DGAT1 and FL-DGAT2. The expression of FL-DGAT1 and FL-DGAT2 was confirmed by probing with anti-FLAG, though it was not clear why less DGAT2 was detected under boiled condition. One possibility is that some DGAT2 precipitated due to the boiling (Figure 4.13). Lipid synthesis was then compared among HEK-293T cells transfected with LacZ, FL-DGAT1, FL-DGAT2 and FL-DGAT1&FL-DGAT2 in presence or absence of oleic acid.

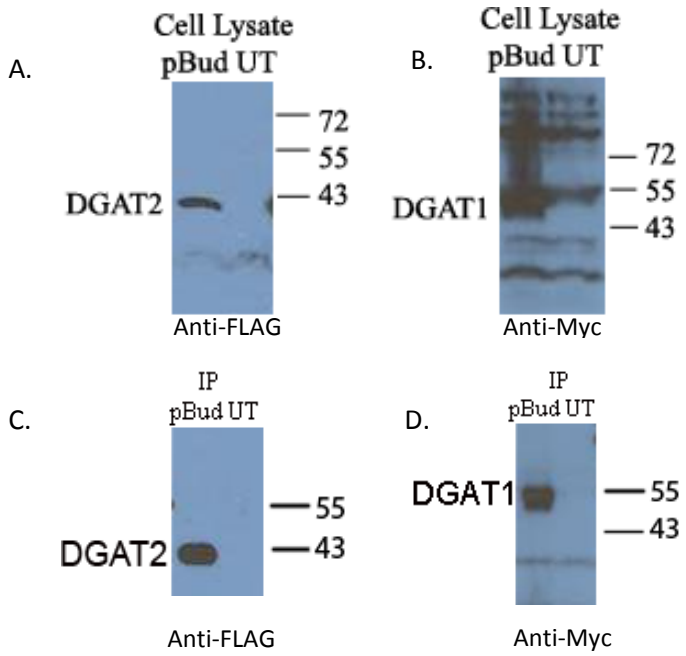


Figure 4.12. DGAT1 and DGAT2 interact. Immunoblot analysis for COS7 cells transfected with pBud vector containing both Myc-DGAT1 and FL-DGAT2 and untransfected cells. (A) Cell lysates were separated by electrophoresis and immunoblotted with anti-FLAG antibody. (B) Same samples were separated and immunoblotted with anti-Myc antibody. (C) FL-DGAT2 was immunoprecipitated by using anti-FLAG, and FL-DGAT2 immunoprecipitates were immunoblotted with anti-FLAG. (D) FL-DGAT2 immunoprecipitates were re-immunoblotted with anti-Myc for immunoblot analysis. UT, untransfected cells. pBud, vector containing Myc-DGAT1 and FL-DGAT2. IP, immunoprecipitation.

Lipids were extracted and separated by TLC (thin layer chromatography). TG levels in different conditions were converted to values and compared (Figure 4.14). FL-DGAT2-transfected cells appeared to synthesize more TGs than untransfected, FL-DGAT1 and LacZ-transfected cells in +/- oleic acid conditions. However, TG levels appeared less in cells co-expressing FL-DGAT1 and FL-DGAT2 compared to single DGAT-transfected cells. This result indicates that co-expression of DGAT1 and DGAT2 does not have an additive effect on TG synthesis in HEK-293T cells, possibly because DGAT1 and DGAT2 were competing for the same pool of substrates.

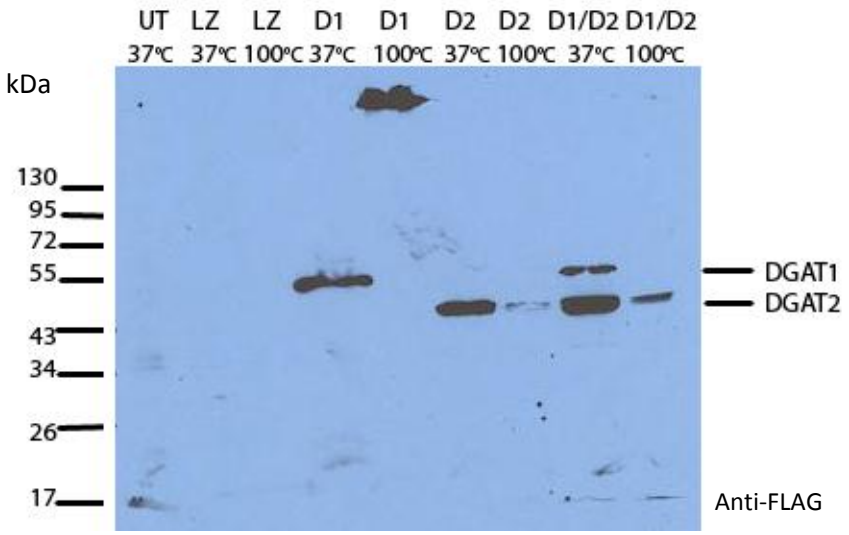


Figure 4.13. Co-expression of FL-DGAT1 and FL-DGAT2 in HEK-293T cells. HEK-293T cells were transiently transfected with LacZ, FL-DGAT1 and/or FL-DGAT2. FL-DGAT1 was prepared under 37°C (to prevent aggregation) and 100°C (as a control). FL-DGAT2 were prepared under 100°C (to prevent aggregation) and 37°C (as a control). They were analyzed by immunoblotting using anti-FLAG antibody. LZ, LacZ transfection. D1/D2, FL-DGAT1 and FL-DGAT2 co-transfection.

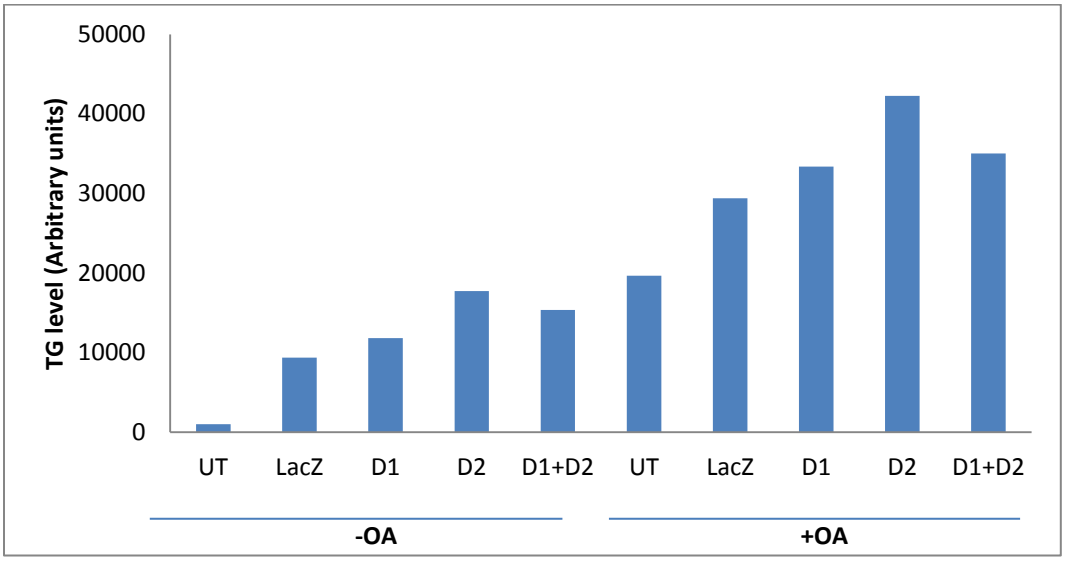


Figure 4.14. Lipid profile of HEK 293T cells overexpressing DGAT1, DGAT2 and DGAT1 and DGAT2. Total lipids were extracted from HEK-293T cells expressing LacZ, FL-DGAT1, FL-DGAT2, FL-DGAT1 and FL-DGAT2 and untransfected cells. Lipid extracts were analyzed by TLC plate with hexane/ethyl ether/acetic acid (80:20:1). TG was quantified by densitometry using ImageJ. +OA, oleic acid treatment. D1+D2, FL-DGAT1 and FL-DGAT2 co-transfection. n=1 sample for each group.

5. DISCUSSION

In previous studies, two DGAT2-interacting proteins have been identified, SCD1 and glycerol phosphate acyl-CoA acyltransferase, GPAT8 (Man *et al*., 2006; Gidda *et al*., 2011). In this study, we identified two proteins that interacted with DGAT1 — DGAT2 and ACAT1. We showed that the co-expression of DGAT1 and DGAT2 did not have a synergistic effect on TG synthesis. We also found that knockdown or overexpression of ACAT1 could decrease or increase TG and cholesterol ester synthesis, respectively.

5.1 Different Functional Roles of DGAT1 and DGAT2

We demonstrated that murine DGAT1 could interact with murine DGAT2. Combined with the topological models of DGAT1 and DGAT2 shown in Fig. 2.5 and Fig.2.6, it is possible that the N-terminus of DGAT1 interacts with C-terminus of DGAT2 as both of these regions are exposed to the cytoplasmic side of the ER. However, it should be noted that in some organisms this interaction may not happen since it has been reported that tung tree DGAT1 and DGAT2 showed distinct, non-overlapping subcellular locations in the ER of tobacco BY-2 cells (Shockey *et al*., 2006). Therefore, if tung tree DGAT1 and DGAT2 distribute separately, it is unlikely that they interact with each other.

We expected that the co-expression of DGAT1 and DGAT2 would increase TG synthesis, since DGAT1 or DGAT2 overexpression did. But our experiments showed that the co-expression of DGAT1 and DGAT2 did not increase TG synthesis. It seemed that the interaction of DGAT1 and DGAT2 disrupted TG synthesis. One possible explanation was that DGAT1 and DGAT2 compete for the same pool of substrates, decreasing the substrate availability for each enzyme. Interestingly, DGAT2's active site is oriented towards the cytosol and DGAT1's is oriented in the ER lumen (Stone *et al*., 2006; Joyce *et al*., 2000). Therefore, fatty acyl moieties in the cytosol have to come across the ER membrane to access the active site of DGAT1.

Perhaps DGAT1 and DGAT2 are able to regulate the enzyme activities of each other as well. For instance, DGAT1 may be able to inactivate DGAT2. When DGAT1 is up-regulated, DGAT1 could suppress the activity of DGAT2. On the contrary, when DGAT2 was up-regulated, it could inactivate DGAT1. This might be an effective measure to control lipid

metabolism and meet different physiological needs. When under stress or fasting, TGs from the liver are incorporated into the VLDL with assistance from DGAT1 for secretion. Although the role of DGAT1 on VLDL assembly is still controversial, it is logical that DGAT1 is more capable for re-esterifying and incorporating hydrolyzed TGs into the VLDL than DGAT2, as it possesses both MGAT and DGAT activities. Also, the active site of DGAT1 is in the ER lumen where VLDLs are assembled. Hence, if DGAT1 prevailed as a response to fasting, it could inactivate and prevent DGAT2 from packaging TGs back into cytosolic lipid droplets for storage. On the contrary, DGAT2 could be responsible for *de novo* TG synthesis and TG storage. The active site of DGAT2 is oriented towards the cytosol where lipid droplets distributed. This mechanism might be pivotal for adipose tissue to be able to store lipids. Taken together, it is reasonable that DGAT1 and DGAT2 could regulate each other to serve different physiological functions.

5.2 DGAT1 and ACAT1 May Be in the Same Protein Complex

We also found that DGAT1 interacted with ACAT1. Before further discussing what this interaction might imply, a brief introduction on ACAT enzymes would be helpful.

Two ACAT genes have been identified, ACAT1 and ACAT2 (Chang *et al*., 1993; Anderson *et al*., 1998; Cases *et al*., 1998b; Oelkers *et al*., 1998). ACAT enzymes are integral membrane proteins that catalyze the synthesis of cholesterol ester from cholesterol and fatty acids (Lin *et al*., 1999; Joyce *et al*., 2000). ACAT1 is present in the ER and contains nine transmembrane domains, with its N-termini facing the cytosol and C-termini in the ER lumen (Guo *et al*., 2005). ACAT2 also resides in ER and contains two transmembrane domains, with both of its N- and C-terminus facing the ER lumen (Lin *et al*., 2003). ACAT1 and ACAT2 share a high degree of homology (Anderson *et al*., 1998). Despite having so many similarities, ACAT enzymes are suggested to have different functions. In African green monkeys, ACAT1 is expressed ubiquitously while ACAT2 is expressed mainly in the liver and small intestine (Lee *et al*., 2000). In humans, ACAT1 accounts for most of the ACAT activity in the liver, while ACAT2 is expressed in a high level in the small intestine (Chang *et al*., 1998). $ACAT1^{-/-}$ mice exhibited attenuated atherosclerosis but had cutaneous xanthomatosis and hyperlipidemia (Yagu *et al*., 2000). In contrast, $ACAT2^{-/-}$ mice showed reduced dietary cholesterol absorption with a

cholesterol-fed diet (Repa *et al*., 2004). These functional studies suggested that ACAT1 was involved in intracellular cholesterol homeostasis, whereas ACAT2 was involved in dietary cholesterol absorption and redistribution. In addition, DGAT1, ACAT1 and ACAT2 are closely related and in the same gene family. DGAT1 and ACAT1 share 15-20% identity and both of them could form dimers and tetramers (ACAT1 could also form hetero-oligomers) (Hofmann, 2000; Yu *et al*., 2002b; Yu *et al*., 1999; Kawasaki *et al*., 1998). DGAT1 and ACAT1 both have active sites facing the cytosolic side of the ER (Joyce *et al*., 2000).

Our experiments demonstrated that disruption of ACAT1 gene expression with siRNA oligonucleotides decreased both cholesterol ester and TG synthesis in HEK-293T cells. This result agreed with previous *in vivo* studies that showed that ACAT1 deficient mice had decreased cholesterol ester and TG synthesis in the liver (Fazio *et al*., 2001; Meiner *et al*., 1996; Meiner *et al.*, 1998). Moreover, the overexpression of ACAT1 in HEK-293T cells increased both cholesterol ester and TG levels. Why does ACAT1 knockdown or overexpression influence TG synthesis, as ACAT1 has no DGAT activity and DGAT1 lacks ACAT activity? We favor a model where ACAT1 and DGAT1 interact as part of a lipid-synthesizing complex producing both TG and cholesterol esters. When ACAT1 is overexpressed, more functional ACAT1-DGAT1 protein complexes are generated, which results in up-regulated cholesterol ester and TG levels. On the other hand, DGAT1 could also affect cholesterol ester synthesis. DGAT1 deficient mice exhibited decreased cholesterol ester levels in the liver and other tissues (Chen *et al*., 2002c; Streeper *et al*., 2006). In mouse macrophages lacking DGAT1, sterol ester levels were reduced (Harris *et al*., 2011). Our experiment showed that the overexpression of DGAT1 increased cholesterol ester synthesis. Therefore, these results further support the concept that ACAT1 and DGAT1 interact as part of a lipid-synthesizing complex. If DGAT1 and ACAT1 could be attached with different fluorescent tags, the condition of their co-localization could be detected. Combination of the usage of compartment markers for the ER or lipid droplets would further reveal their distribution and how their interaction affects their distribution.

Perhaps, the interaction of DGAT1 and ACAT1 and its effects can help us understand VLDL secretion in the perspective of a DGAT1-ACAT1 protein complex. It is well documented that ACAT1 overexpression increases plasma VLDL levels, and ACAT inhibitors decrease plasma VLDL levels (Liang *et al*., 2004; Taghibiglou *et al*., 2001; Leon *et al*., 2005; Burnett *et*

al., 1999). Other studies showed that long-term (12 days) overexpression of human DGAT1 in hepatoma cells or in mice could increase the secretion of VLDL (Yamazaki *et al*., 2005; Millar *et al*., 2006). But, DGAT1 deficiency in mice did not affect VLDL secretion, and macrophages deficient in both DGAT enzymes could form lipid droplets with cholesterol-rich lipoproteins (Harris *et al*., 2011; Liu *et al*., 2008). Taken together, we speculate that an ACAT1-DGAT1 protein complex promotes VLDL synthesis and secretion. In addition, mass spectrometry of DGAT1 immunoprecipitates revealed a protein capable of reducing cholesterol from its oxidized form, 24-dehydrocholesterol reductase (DLCR) (Waterham *et al*., 2001). This suggested that ACAT1 also had the ability to associate with other proteins, and thus the role of ACAT1 in the complex was an important one.

Unfortunately, we could not identify the interacting regions between DGAT1 and ACAT1. Using DGAT1 mutants, we only found that the N-terminus of DGAT1 was not responsible for its interaction with ACAT1. Thus, a region in the C-terminus of DGAT1 likely interacts with ACAT1.

5.3 Role of FAR1

By mass spectrometry, fatty acyl-CoA reductase 1 (FAR1) was identified as a potential DGAT1-interacting protein. Unfortunately, we could not confirm this interaction. FAR1 reduces fatty acyl-CoA to synthesize fatty alcohol (Cheng and Russell, 2004a; Cheng and Russell, 2004b). It localizes to the peroxisome and prefers fatty acids with 16 or 18 carbons, which are the preferred substrates of DGAT1. In mammals, FAR1 is ubiquitously expressed. High FAR1 mRNA levels are found in organs synthesizing wax esters and ether lipids, such as the brain and sebaceous glands (Wanders and Waterham, 2006). Specifically, as DGAT1 possesses wax synthase activity and FAR1 is capable of supplying fatty alcohol, we hypothesized that they might interact. However, our experiments showed that although FAR1 was a potential binding partner of DGAT1 as identified by mass spectrometry, FAR1 could not be co-immunoprecipitated with DGAT1 indicating that these two proteins do not interact. Moreover, DGAT1 and FAR1 are present in different subcellular compartments. DGAT1 resides in the ER and FAR1 is localized to peroxisomes (Stone *et al*., 2009; Cheng and Russell, 2004a).

5.4 TG Synthetase Complex

In order to increase local substrate concentration and allowing serial biochemical reactions to proceed efficiently, a protein complex is preferred for catalyzing a series of reactions. A TG-synthetase complex is proposed in Figure 5.1.

While DGAT1 and DGAT2 interact and may appear in the same TG-synthetase complex, it is possible that DGAT1 and DGAT2 could perform different functional roles. I propose that the two DGAT enzymes are able to group with different protein partners in a nonsimultaneous manner. Particularly, when DGAT1 associates with its protein partners, DGAT2 cannot form a complex with other protein partners except for DGAT1. On the other hand, when DGAT2 is grouped with its protein partners, DGAT1 cannot complex with other protein partners except for DGAT2. Therefore, there may be heterogeneous TG-synthetase complexes associating with different proteins to perform distinct functions. DGAT1 would associate with enzymes responsible for lipid re-esterification and redistribution, such as ACAT1 and MGAT. One TG-synthetase complex found in the rat intestinal cells possessed acyl-CoA ligase (ACL), AAT, MGAT and DGAT activities (Lehner and Kuksis, 1995). We suspect that the core DGAT enzyme involved in this complex was DGAT1. Firstly, DGAT1 possesses MGAT activity and is more dependent on dietary diacylglycerol from hydrolyzed fats (Yen *et al*., 2005). Also, in the same study, one protein associated with AAT was 54kDa, which is about the size of DGAT1. Third, according to the fact that DGAT1 deficient mice exhibit delayed chylomicron secretion compared to wild-type mice, DGAT2 was suggested to interact with another diacylglycerol transacylase instead of MGAT in this case (Buhman *et al*., 2002).

In contrast, DGAT2 is responsible for bulk TG synthesis. Thus, it is likely complexed with proteins involved in *de novo* TG biosynthetic pathway such as GPAT, LPAT and PAP. A cytosolic TG-synthetase complex was demonstrated in *Rhodotorula glutinis* (Gangar *et al*., 2001). It contained LPAT, PAP, DGAT, acyl-acyl carrier protein synthetase (AACS) and an acyl carrier protein. The main enzymes in *de novo* TG synthetic pathway were present, and we speculate that DGAT2 (specifically, its ortholog in yeast) was the core in this protein complex. DGAT2 appears to use endogenous fatty acids and associated proteins supplying them, such as SCD1. Therefore, the TG-synthetase complex with high ability for *de novo* TG synthesis is likely to employ DGAT2 as the core protein to perform its function.

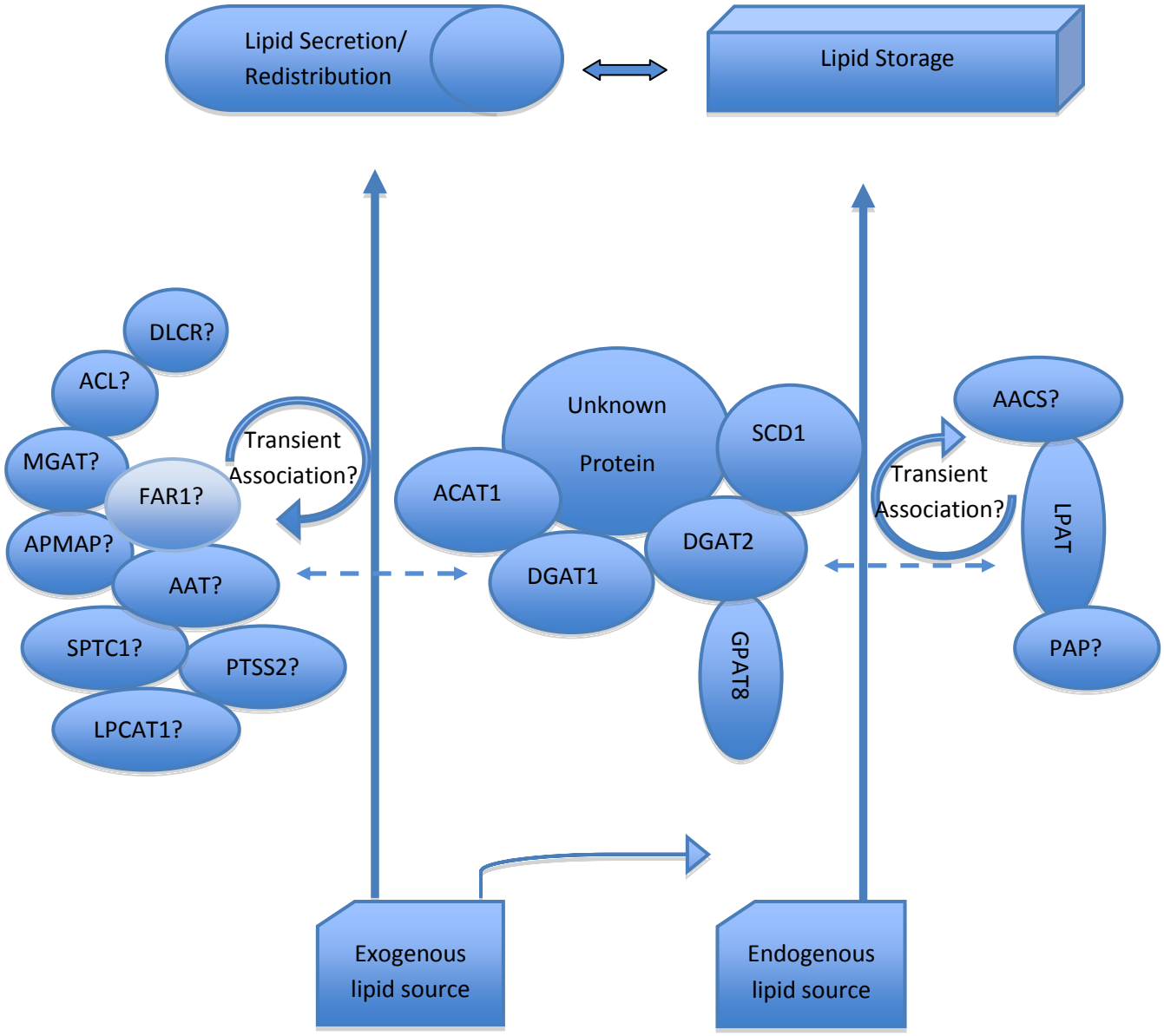


Figure 5.1. Proposed TG synthetase complexes. DGAT1 and DGAT2 transiently group with different protein partners and determine the functions of the complex. The function of DGAT1 complex might be involved in lipid redistribution with using exogenous (dietary) lipids. DGAT2 complex might mainly store lipids using both exogenous and endogenous lipids. Two pools of lipids could be transferred from each other. The question mark refers to the proposed interaction and other unknown proteins that may associate with DGAT enzymes. DLCR, 24-dehydrocholesterol reductase. ACL, acyl-CoA ligase. AACS, acyl-acyl carrier protein synthetase. APMAP, adipocyte plasma membrane-associated protein. AAT, acyl-acyl transferase. SPTC1, serine palmitoyltransferase 1. PTSS2, Phosphatidylserine synthase 2. LPCAT1, lysophosphatidylcholine acyltransferase 1. SCD1, stearoyl-CoA desaturase 1. GPAT8, glycerol phosphate acyl-CoA transferase 8. LPAT, lysophosphatidate acyl transferase. PAP, phosphatidate phosphatase.

TG-synthetase complex might determine the direction of lipid synthesis, such as from diacylglycerol to TGs, or from diacylglycerol to phospholipids. The down-regulation of DGAT enzymes, which would suppress the TG-synthetase complex, could promote the lipid efflux from diacylglycerol to phospholipids instead of TG. During cell division, DGAT1 and DGAT2 mRNA levels were suppressed by the MEK-ERK pathway, which was responsible for decreasing the secretion of VLDL (TG-rich lipoprotein) and TG synthesis (Tsai *et al*., 2007; Wang *et al*., 2010). This implied that under cell division, diacylglycerol were used for the synthesis of phospholipids instead of TG. On the other hand, the up-regulation of DGAT enzymes that resulted in more TG-synthetase complexes were likely capable of shifting the flux of lipids from phospholipids to TG.

Thirdly, in the TG-synthetase complex, DGAT-interacting proteins might provide signals for different cellular activities. We speculated that two proteins revealed from mass spectrometry could perform this function. A plasma membrane-associated protein (APMAP) was shown to interact with DGAT1. Current studies show that APMAP localizes to the ER in preadipocytes and translocates to the plasma membrane during adipogenesis (Bogner-Strauss *et al*., 2010). The silencing of APMAP can impair adipogenesis. Enforced overexpression of APMAP, however, cannot initiate the translocation of APMAP to plasma membrane in preadipocytes. Therefore, the translocation of APMAP may need the up-regulation of DGAT1, as does DGAT1 during adipogenesis. If enforced co-expression of DGAT1 and APMAP in preadipocytes could promote APMAP translocate to the plasma membrane, this potential interaction of DGAT1 and APMAP could be indirectly identified and important for adipogenesis. In addition, DGAT1 could possibly also interact with serine palmitoyltransferase 1, which synthesizes sphinganine, a precursor of ceramide synthesis. The latter can be attached onto sugar molecules to transmit various cell-signals, such as differentiation, proliferation or apoptosis. Thus, the potential interaction between DGAT1 and serine palmitoyltransferase 1 may serve to provide different cell signals.

Furthermore, from mass spectrometry of DGAT1 immunoprecipitates, we observed phosphatidylserine synthase 2 (PTSS2), serine palmitoyltransferase 1 (SPTC1) and lysophosphatidylcholine acyltransferase 1 (LPCAT1) (Table 3). All these proteins localize to the ER as DGAT1 does, which increases the chances for them to interact with DGAT1 and affect the balance of TG and their product formation. Specifically, LPCAT1 could synthesize

phosphatidylcholine that forms the lipid membrane layer. LPCAT has been reported to surround the lipid droplets and is hypothesized to assist the expansion of the lipid droplet membrane independent of an ER connection (Moessinger *et al*., 2011). TG-synthetase likely interacts with LPCAT1 to incorporate TG into lipid droplets. If the co-immunoprecipitation of LPCAT1 could reveal DGAT1, it would help us further understand the formation of lipid droplets.

5.5 Future Directions

Our experiments have studied the relationship between DGAT1 and DGAT2 as well as DGAT1 and ACAT1. Further studies might include investigating the hypothesis that DGAT1 and DGAT2 serve different functional roles. We are also interested in finding out other effects of DGAT1 and ACAT1's interaction, and better understanding the biochemical significance of this interaction, such as VLDL formation. We could determine the possible interactions between DGAT1 and other lipid-synthesizing proteins, and explore the formation of lipid droplets and whether these possible interactions could divert the lipid synthesis direction.

To determine whether DGAT1 and DGAT2 can regulate the activities of each other, we could identify interacting regions of DGAT1 with DGAT2 by constructing DGAT1 truncated mutants (without the C-terminus or middle region). Truncated DGAT1 mutants without interacting regions would be expected to be incapable of binding to DGAT2 and would not be co-immunoprecipitated. Thus interacting regions of DGAT1 and DGAT2 could be identified. We already found that the co-transfection of DGAT1 and DGAT2 could not increase TG synthesis. Therefore, DGAT1 might be able to regulate the enzyme activity of DGAT2. To verify this hypothesis, we could design mutant DGAT1 unable to associate with DGAT2 by deleting its DGAT2-interacting region, and the mutant DGAT1 would be co-transfected with normal DGAT2. If the co-transfection increased TG synthesis, it would suggest that mutant DGAT1 could no longer regulate, specifically inhibit, DGAT2 and thus TG synthesis increased. Further, the enzyme kinetics of DGAT2 could be determined when DGAT2 was co-transfected with mutant DGAT1 without DGAT activity constructed by site-directed mutagenesis. If DGAT2's affinity for substrates decreased, the hypothesis is valid. It would further demonstrate that DGAT1 and DGAT2 had different functional roles in TG synthesis.

We have shown that DGAT1 and ACAT1 overexpression increases cholesterol ester and TG synthesis, respectively. Next we could identify the interacting regions residing in DGAT1 and ACAT1 sequence, and this could provide information of DGAT1 and ACAT1's effects on VLDL secretion. As reviewed, ACAT1 was positively related to plasma VLDL (Liang *et al*., 2004; Wilcox *et al.*, 1999; Burnett *et al*., 1997). In contrast, DGAT1 seemed only to facilitate VLDL secretion (Yamazaki *et al*., 2005; Millar *et al*., 2006; Liu *et al*., 2008). We could determine the interacting regions of DGAT1 and ACAT1 by designing truncated DGAT1 or ACAT1. After identifying the interacting region, mutant DGAT1 unable to interact with ACAT1 could be used for co-expression with ACAT1. If the co-expression of mutant DGAT1 and normal ACAT1 did not increase plasma VLDL or the TG content of VLDL, it would suggest that DGAT1 could assist ACAT1 for VLDL secretion. In addition, obesity was shown associated with high plasma cholesterol levels. We could compare cholesterol ester and cholesterol levels between cells co-expressing DGAT1 mutant with ACAT1 and normal DGAT1 with ACAT1. If mutant DGAT1 and ACAT1 overexpression showed decreased cholesterol ester content in lipid droplet, it would suggest that the interaction of DGAT1 and ACAT1 is significant for cholesterol ester incorporation into lipid droplets. That would further imply that the interaction of DGAT1 and ACAT1 is crucial for cholesterol ester storage and plasma cholesterol balance inside body.

In addition, we could also determine the relationships between DGAT enzymes and proteins responsible for phospholipid and sphingolipid synthesis revealed by mass spectrometry of DGAT1 immunoprecipitates. Do these proteins compete for substrate (acyl moieties) with DGAT enzymes or DGAT enzymes utilize phospholipids as acyl donor to generate TG? On one hand, our experiment has demonstrated that DGAT1 and DGAT2 might compete for the same pools of substrates, which suggests that DGAT1-associating proteins could have a competing relationship with DGAT1. On the other hand, in yeast and plant a phospholipid acyltransferase uses phospholipids as acyl donors to produce diacylglycerol, which are substrates for DGAT enzymes (Besterman *et al*., 1986). It may be that both situations exist, but whether DGAT-associating proteins are acyl competitors or acyl donors depends on the actual physiological needs. We could use co-immunoprecipitation technique to determine the interactions between these proteins and DGAT enzymes. Furthermore, we could overexpress or silence them to

examine their effects on TG production and other lipid synthesis. These studies could reveal the functional roles of the proteins.

6. REFERENCES

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