Regulation of Acyl-CoA:Diacylglycerol Acyltransferase-1 by Protein Phosphorylation

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ABSTRACT

Triacylglycerols are the predominant molecules of energy storage in eukaryotes. Triacylglycerol synthesis is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2. Although the use of molecular tools, including targeted disruption of either DGAT enzyme, has shed light on their metabolic functions, little is known about the mechanisms responsible for regulating DGAT activity. Several lines of evidence from previous studies have suggested that DGAT1, but not DGAT2, is subject to regulation by phosphorylation and that protein kinase A (PKA)-dependent pathways are likely involved. In this study, the role of PKA in regulating DGAT activity and triacylglycerol synthesis during lipolysis was investigated. By using 3T3-L1 adipocytes, *in vitro* DGAT activity was shown to increase 2 fold during lipolysis. This data suggests that PKA might phosphorylate and activate DGAT1 during lipolysis to promote the recycling/re-esterification of excessive free fatty acids into triacylglycerols before they reach toxic levels within the cell. Additionally, high-performance liquid chromatography – electrospray ionization – mass spectrometry/mass spectrometry was exploited to identify PKA phosphorylation sites of DGAT1, and serine-17, -20 and -25 were identified as potential PKA phosphorylation sites using this methodology. The functional importance of these three potential phosphorylation sites was examined. Mutations of these sites to alanines (to prevent phosphorylation) or aspartates (to mimic phosphorylation) gave rise to enzymes functioning similarly to wild-type DGAT1. These phosphorylation sites appeared to be functionally silent as they were not involved in regulating DGAT1 activity, multimer formation, or enzyme stability. However, PKA phosphorylation at these three sites seemed to play a role in affinity of DGAT1 for its diacylglycerol substrate. These results indicate the existence of other unidentified, functionally active PKA phosphorylation sites or phosphorylation sites of other kinases, which are involved in regulating DGAT1.

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To my parents,

whose love and support made this thesis possible

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LIST OF ABBREVIATIONS

Acyl-CoA: Diacylglycerol Acyltransferase	DGAT
Acyl-CoA: Cholesterol Acyltransferase	ACAT
Acyl-CoA: Retinol Acyltransferase	ARAT
Adipose Triacylglycerol Lipase	ATGL
AMP-activated Protein Kinase	AMPK
Apolipoprotein-B	ApoB
Bovine Serum Albumin	BSA
Chlorophenylthio-3'-5'-Cyclic Adenosine Monophosphate	CPT-cAMP
3'-5'-Cyclic Adenosine Monophosphate	cAMP
Dimethyl Sulfoxide	DMSO
Dulbecco’s Modified Eagle's Medium	DMEM
Electrospray Ionization	ESI
Endoplasmic Reticulum	ER
Fetal Bovine Serum	FBS
High-Performance Liquid Chromatography	HPLC
Hormone Sensitive Lipase	HSL
Human Embryonic Kidney 293T	HEK293T
Luria-Bertani	LB
Mass Spectrometry	MS
Mitochondria-Associated Membranes	MAM
Phosphate-Buffered Saline	PBS
Protein Kinase A	PKA
Room Temperature	RT
Synthetic Complete Medium Lacking Uracil	SC-Ura medium
Terrific Broth	TB
Thin Layer Chromatography	TLC
Very Low Density Lipoproteins	VLDL

Amino Acid Abbreviations

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

1.0 INTRODUCTION

As a result of our society obtaining overly rich nutrition and our preponderance to sedentary lifestyles, obesity has rapidly become a global health issue. In many incidences of obesity, individuals also develop insulin resistance, which can lead to type 2 diabetes mellitus. These conditions in turn predispose to hyperlipidemia and atherosclerotic coronary heart disease. These associated conditions—obesity, insulin resistance, and atherosclerosis—are components of the metabolic syndrome, which is one of the most prevalent non-communicable diseases and poses a significant threat to public health (Zimmet *et al.*, 2001). Therefore, basic research on the pathophysiological mechanisms underlying obesity and obesity-associated diseases is essential for achieving effective interventions for these diseases.

Triacylglycerols are a class of neutral lipids that represent the major storage form of energy in eukaryotic organisms. However, an imbalance between energy intake and expenditure can lead to excessive accumulation of triacylglycerols in adipose tissue resulting in obesity. Excessive accumulation of triacylglycerols in non-adipose tissues, such as skeletal muscle, liver, heart, and pancreatic beta cells, is associated with insulin resistance and can lead to type 2 diabetes mellitus and cardiovascular disease (Unger, 2002; Friedman, 2002).

The final step of triacylglycerol biosynthesis is catalyzed by the microsomal enzyme acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). Two DGAT enzymes have been identified (DGAT1 and DGAT2), and studies have clearly demonstrated their unique roles in lipid metabolism (Smith *et al*, 2000; Chen *et al*., 2002a; Chen *et al*., 2003; Stone *et al*., 2004; Wang *et al*., 2007). However, the mechanisms by which these enzymes are regulated are poorly understood. DGAT1 has the consensus sequences for two protein kinase A (PKA) phosphorylation sites that are not present in DGAT2. In previous studies, only DGAT1, but not DGAT2, appeared to be phosphorylated (Stone *et al.*, unpublished observations). Using a candidate kinase approach, PKA has been identified as one possible kinase that could phosphorylate DGAT1 and inhibit its *in vitro* activity. This preliminary data indicates that DGAT1, but not DGAT2, is subject to regulation by phosphorylation and that PKA-dependent pathways are likely involved. We hypothesize that during lipolysis in adipose tissue, PKA would phosphorylate and inactivate DGAT1, which in turn would facilitate the efficient net release of stored energy by decreasing triacylglycerol synthesis.

The objective of my thesis was to examine the role that PKA has in regulating DGAT1 activity and triacylglycerol synthesis during lipolysis. Additionally, my goal was to identify PKA phosphorylation sites of DGAT1 by mass spectrometry (MS) and determine their functional importance.

2.0 LITERATURE REVIEW

2.1 Triacylglycerols

Triacylglycerols are a class of neutral lipids and are the predominant storage form of metabolic energy in eukaryotic organisms (Coleman *et al*., 2003; Hertzel *et al*., 2008; Yen *et al*., 2008). Triacylglycerols are composed of three fatty acids attached to a glycerol backbone by ester bonds. Various triacylglycerols possess different physical and chemical properties, depending on their fatty acid compositions. Triacylglycerols containing primarily saturated long-chain fatty acids tend to be solid at body temperature, and those containing unsaturated or short-chain fatty acids tend to be liquid. Triacylglycerols are highly reduced molecules and can be stored in large quantities as they are anhydrous. The highly reduced hydrocarbon tail of the fatty acid makes triacylglycerols an efficient fuel storage that can be readily oxidized to produce large quantities of ATP. Moreover, the very hydrophobic nature of the hydrocarbon tail precludes concomitant storage of excess water, which in turn minimizes mass and spatial requirements of the organism. The relatively straight, chain-like structure of the fatty acid further facilitates the dense packing of many molecules into each cell, maximizing the efficiency of energy storage. Therefore, triacylglycerols store 6-fold more energy than the same amount of hydrated glycogen (Yen *et al*., 2008). In plants, triacylglycerols are particularly abundant in oil-rich seeds and fruits, developing microspores and pollen grains (Murphy and Vance, 1999; Xu *et al*., 2008). Triacylglycerols in microorganisms are present in a small number of cytosolic lipid droplets that also contain sterol esters (Murphy and Vance, 1999). In mammals, adipose tissue serves as the major energy storage depot in the body and provides thermal insulation for organisms (Yen *et al*., 2008). A typical adipocyte from adipose tissue contains one or a few large cytoplasmic triacylglycerol-rich lipid droplets that accounts for nearly the entire volume of the cell (Murphy and Vance, 1999). Energy stored in the form of triacylglycerols can be utilized through lipolysis, the process by which triacylglycerols are hydrolyzed to glycerol and fatty acids. Released fatty acids and glycerol enter the circulation and are utilized by tissues that require energy. Triacylglycerols are also abundant in mammalian hepatocytes and enterocytes. In addition to energy storage, in mammalian enterocytes and hepatocytes, triacylglycerols can be synthesized, assembled and secreted into

the circulation as chylomicrons from the intestine or very low-density-lipoproteins from the liver, which distribute dietary and endogenously synthesized fatty acids to peripheral tissues (Coleman *et al*., 2003; Yen *et al*., 2008). Furthermore, triacylglycerol biosynthesis serves to protect cells from the potentially membrane-damaging effects of excessive fluxes of fatty acids and acyl-CoAs, because their incorporation into triacylglycerols converts them to a non-toxic compound that can be safely stored (Coleman *et al*., 2003).

Although triacylglycerols play a variety of critical roles for normal physiology of eukaryotic organisms, an imbalance between energy intake and expenditure can lead to excessive accumulation of triacylglycerols in adipose tissue resulting in obesity. Excessive accumulation of triacylglycerols in non-adipose tissues, such as skeletal muscle, liver, heart, and pancreatic beta cells, is associated with insulin resistance and can lead to type 2 diabetes mellitus, which in turn predispose to hyperlipidemia and atherosclerotic coronary heart disease (Unger, 2002; Friedman, 2002).

2.2 Triacylglycerol Biosynthesis and DGAT Enzymes

Triacylglycerol biosynthesis occurs mainly via two acyl-CoA dependent pathways: the Kennedy (or glycerol phosphate) pathway and the monoacylglycerol pathway (Lehner *et al*., 1996; Yen *et al*., 2008) (Figure 2.1). The glycerol phosphate pathway, which functions in most cell types, represents the *de novo* route to triacylglycerol formation. On the other hand, the monoacylglycerol pathway accounts for the majority of triacylglycerol synthesis (~80%) in the small intestine, where most dietary triacylglycerols are absorbed as fatty acids and monoacylglycerols that require re-assembly to triacylglycerols (Buhman *et al*., 2002). The final step of both pathways is catalyzed by DGAT enzymes. DGAT catalyzes the formation of an ester bond between a long chain fatty acyl-CoA and the free hydroxyl group of sn-1,2 (2,3)-diacylglycerol, but not sn-1,3-diacylglycerol, generating triacylglycerol (Lehner *et al*., 1993) (Figure 2.2). It is important to note that the diacylglycerol synthesized from the monoacylglycerol pathway and the glycerol phosphate pathway are not metabolically equivalent, because the former is directed to triacylglycerol synthesis only whereas the latter may be utilized to synthesize either triacylglycerol or phospholipids (Charles *et al*., 2007). The DGAT reaction is believed to occur mainly at the endoplasmic reticulum (ER), and newly

synthesized triacylglycerols are thought to be channeled either into cytosolic lipid droplets for storage or triacylglycerol-rich lipoproteins for secretion into the circulation (Yen *et al*., 2008). In addition to the two acyl-CoA dependent pathways of triacylglycerol biosynthesis, an acyl-CoA independent pathway has been reported in mouse small intestine, in which a fatty acyl moiety from one diacylglycerol can be transferred to a second diacylglycerol to synthesize triacylglycerol in a reaction catalyzed by diacylglycerol transacylase (Buhman *et al.*, 2002).

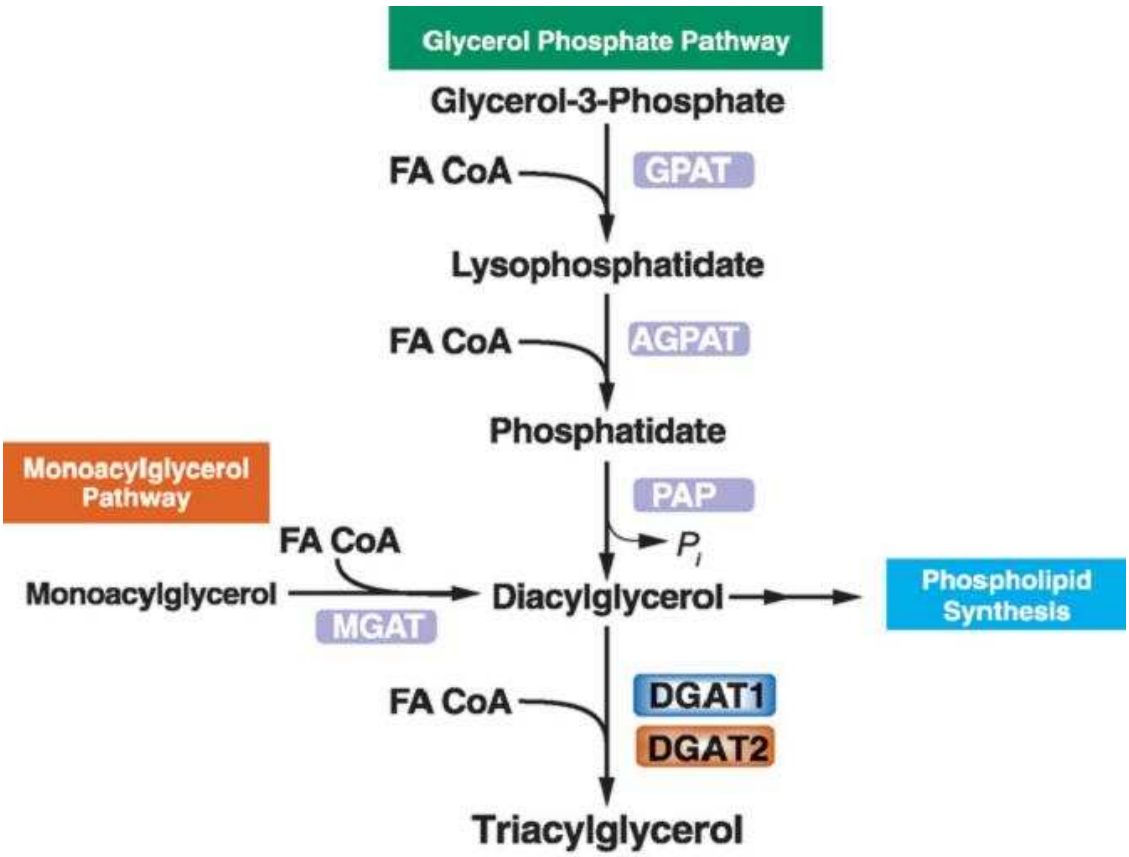


Figure 2.1 Metabolic Pathways of Triacylglycerol Biosynthesis.
Triacylglycerol biosynthesis occurs via two predominant pathways: the Kennedy (or glycerol phosphate) pathway and the monoacylglycerol pathway. DGAT1 and DGAT2 catalyze the final step of triacylglycerol synthesis. GPAT: glycerol-phosphate acyltransferase; AGPAT: acylglycerol-phosphate acyltransferase; PAP: phosphatidic acid phosphohydrolase; MGAT: acyl-CoA:monoacylglycerol acyltransferase. The diagram is taken from Yen *et al.* (2008).

2.3 Distinct Gene Families of DGAT Enzymes

Two distinct DGAT genes have now been identified, *Dgat1* and *Dgat2*, which share no sequence homology. The gene encoding DGAT1 was identified by its sequence similarity to acyl-CoA:cholesterol acyltransferase (ACAT) enzymes, which catalyze cholesterol ester biosynthesis (Chang *et al*., 1993; Cases *et al*., 1998). Both DGAT1 and ACAT enzymes belong to a large family of membrane-bound O-acyltransferases (Figure 2.3), which catalyzes the covalent joining of fatty acyl moieties onto the hydroxyl or thiol groups of lipid and protein acceptors (Kadowaki *et al*., 1996; Zhai *et al*., 2004; Yang *et al*., 2008). The gene encoding DGAT2 was identified by its homology to a DGAT purified from the fungus *Mortierella rammaniana* (Cases *et al*., 2001; Lardizabal *et al*., 2001). DGAT2 belongs to a seven-member gene family including monoacylglycerol acyl-CoA acyltransferase-1, -2 and -3 and wax synthases (Cases *et al*., 2001; Yen *et al*., 2002; Cao *et al*., 2003; Cheng *et al*., 2003; Yen *et al*., 2003; Turkish *et al*., 2005) (Figure 2.3).

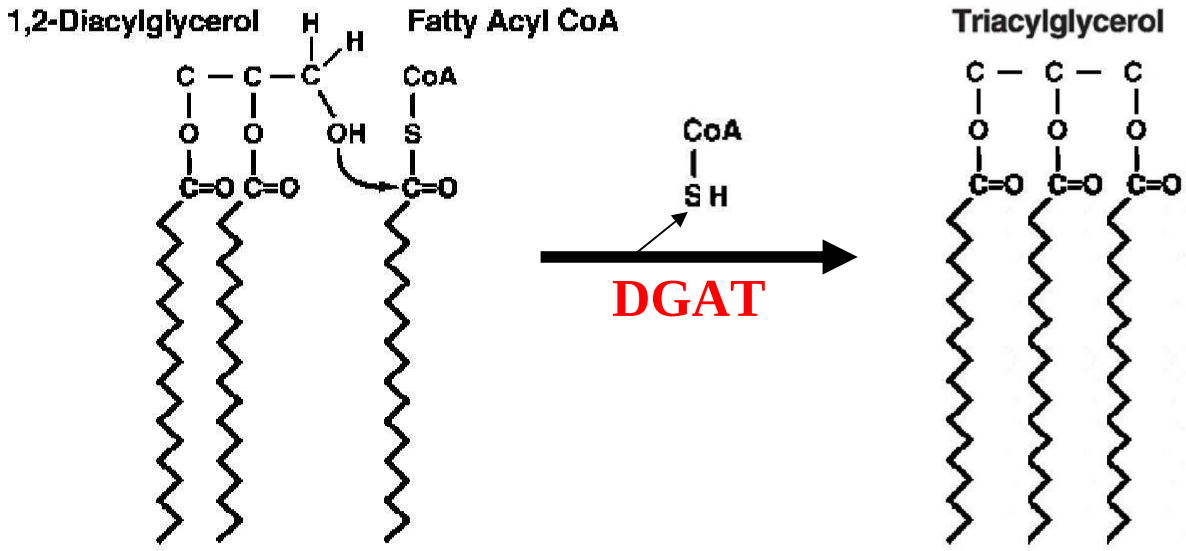


Figure 2.2 DGAT Catalyzed Reaction.
DGAT enzymes catalyze the formation of an ester linkage between a fatty acyl-CoA and the free hydroxyl group of diacylglycerol, generating triacylglycerol. The diagram is modified from Yen *et al*. (2008).

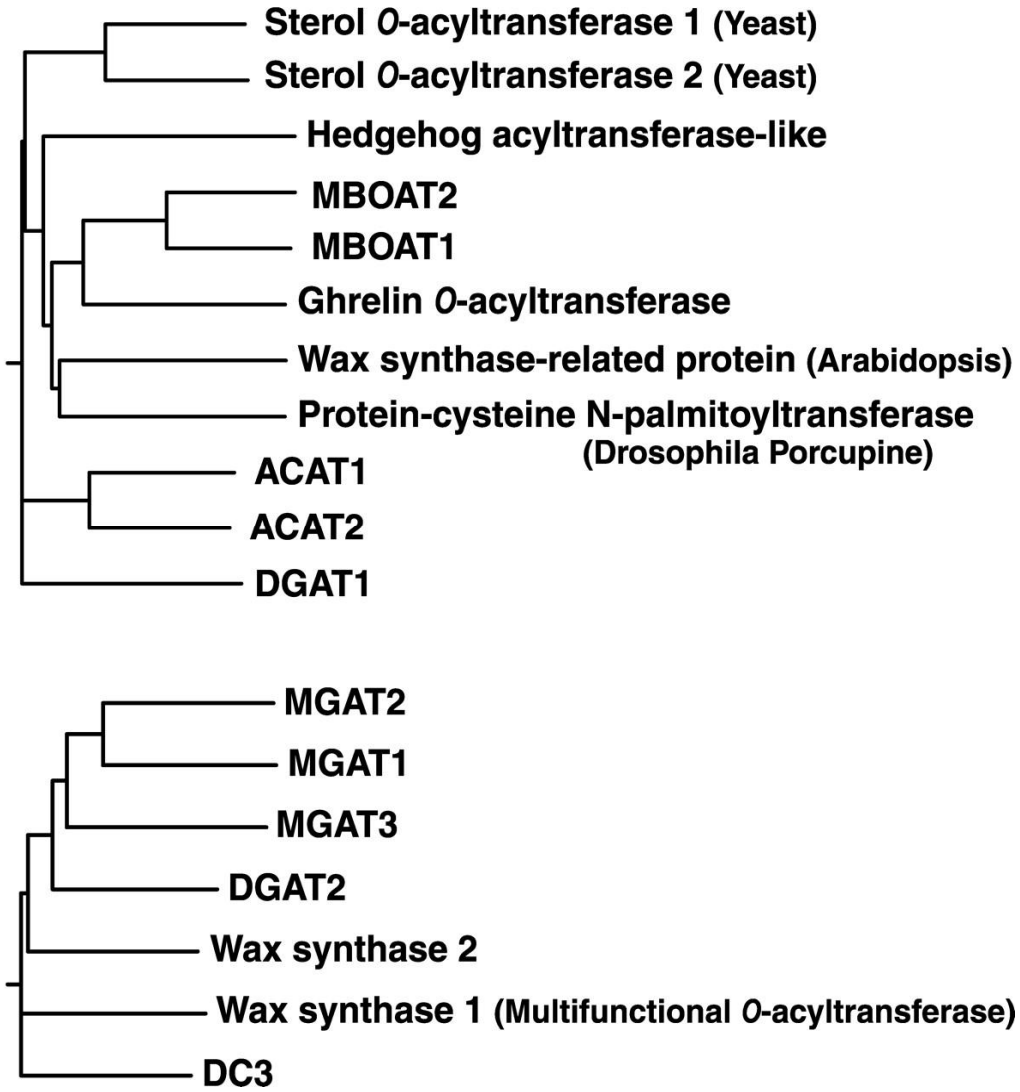


Figure 2.3 Dendrograms of the DGAT Gene Families.
The ACAT/DGAT1 gene family belongs to a superfamily of membrane-bound O-acyltransferases (MBOAT). Selected members are human enzymes unless indicated. MBOAT1 and MBOAT2 are members whose activities are unknown. DGAT2 belongs to a seven-member gene family including monoacylglycerol acyl-CoA acyltransferase (MGAT)-1, -2 and -3 and wax synthases. DGAT candidate 3 (DC3) refers to a family member whose activity is unknown. The diagrams are taken from Yen *et al.* (2008).

2.4 Structural and Functional Aspects of DGAT Enzymes

Due to the difficulties in purifying DGAT enzymes to homogeneity, little is known about the molecular aspects of their structures and functions. Murine and human *Dgat1* genes encode a protein that is ~500 amino acids in length and shares 15-25% sequence identity, mostly in the C-termini, with ACAT enzymes (Cases *et al*., 1998). A highly conserved motif present in DGAT1 and ACAT enzymes (FYXDWWN; amino acids 371–377 of murine DGAT1) has been implicated in binding the substrate fatty acyl-CoA. Mutagenesis of this conserved domain in ACAT1 resulted in decreased enzymatic activity and affinity for acyl-CoA (Guo *et al*., 2001). However, there is no experimental evidence demonstrating the involvement of this domain in substrate binding for DGAT1. In contrast, two independent studies indicated that the fatty acyl-CoA binding site may lie at the N-terminus of DGAT1, since a fragment containing approximately the first 100 amino acids of DGAT1 from the plant *Brassica napus* and mouse directly bind fatty acyl-CoA (Weselake *et al*., 2006; Siloto *et al*., 2008). Toward the C-terminal region, DGAT1 has a putative diacylglycerol-binding motif that is highly conserved across species and also present in various protein kinase C isoforms (Timmers *et al*., 2008; Talharia *et al*., 2009). Protein kinase C binds to diacylglycerol through this motif. DGAT1 is speculated to utilize this domain to interact with one of its substrates, diacylglycerol. However, experimental evidence demonstrating the involvement of this domain in diacylglycerol binding is lacking.

Recently, McFie *et al.* (2010) mapped the membrane topology of murine DGAT1 and demonstrated that it has three transmembrane domains with the N-terminus exposed to the cytosol. The C- terminal region of DGAT1, which accounts for approximately 50% of the protein, was shown to be present in the ER lumen. A highly conserved histidine residue (H426 for murine DGAT1) in DGAT1 and ACAT enzymes, which McFie's model suggested is present in the ER lumen, was determined to comprise part of the active site of DGAT1. Mutagenesis of H426 to alanine resulted in the complete loss of enzymatic activity of DGAT1. Despite the sequence homology between DGAT1 and ACAT enzymes, the putative active histidine residues in ACAT1 (H460) and ACAT2 (H434) were suggested to be partially imbedded within the cytoplasmic side of the lipid bilayer (Lin *et al*., 2003).

Like ACAT enzymes, DGAT1 exists as a homotetramer (Cheng *et al*., 2001), and the N-terminal domain of each subunit may be responsible for regulating dimer/tetramer formation

(Cheng *et al*., 2001; McFie *et al*., 2010).

Murine and human *Dgat2* genes encode a protein that is ~350-400 amino acids in length and shares 40–45% sequence identity with its family members throughout the entire lengths of the proteins (Cases *et al*., 2001; Lardizabal *et al.*, 2001). The highly conserved sequence, "HPHG", is present in all DGAT2 family members and is likely part of the active site of DGAT2 (Stone *et al*., 2006). Mutagenesis of this sequence in murine DGAT2 resulted in significant reduction of *in vitro* DGAT activity. Additionally, DGAT2 contains a consensus sequence (FLXLXXXn, where Xn is a nonpolar amino acid) for a putative neutral lipid binding domain that is present in proteins that bind to or metabolize neutral lipids (Stone *et al*., 2006). DGAT2 may utilize this domain to bind diacylglycerol. Mutations in this region of murine DGAT2 also markedly reduced *in vitro* DGAT activity. Stone *et al* also demonstrated that DGAT2 is an integral membrane protein with two transmembrane domains and that both the N- and C- termini are exposed to the cytosol.

2.5 Enzymology and Cellular Biology of DGAT Enzymes

Although both DGAT1 and DGAT2 have broad substrate specificities and utilize the same long-chain fatty acyl-CoAs (Cases *et al*., 2001), DGAT1, but not DGAT2, possesses additional acyltransferase activities *in vitro* (Yen *et al*., 2005). DGAT1 is a potent acyl-CoA: retinol acyltransferase (ARAT), which catalyzes the synthesis of retinyl esters from retinol and fatty acyl-CoA substrates. Several studies further suggest that DGAT1 may function as an ARAT *in vivo*, especially when the intracellular concentration of unesterified retinols is high (Yen *et al*., 2005; Wongsiriroj *et al*., 2008; Yamaguchi *et al*., 2008). In addition, DGAT1 exhibits *in vitro* acyl-CoA:monoacylglycerol acyltransferase activity, which catalyzes the esterification of monoacylglycerol with a fatty acyl-CoA to produce diacylglycerol (Yen *et al*., 2005). The importance of the MGAT activity for DGAT1 function has not been determined. DGAT1 also displays *in vitro* activity of wax monoester and wax diester synthases, which catalyze the synthesis of wax esters from fatty alcohols and fatty acyl-CoAs (Yen *et al*., 2005).

On the other hand, DGAT2 overexpression in rat hepatoma cells resulted in substantially higher triacylglycerol accumulation than DGAT1 overexpression did, with considerably larger lipid droplets (Stone *et al*., 2004). These findings are consistent with

findings of the two enzymes in yeast. Dga1, the yeast homologue of DGAT2, contributes predominantly to triacylglycerol synthesis and storage in yeast (Oelkers *et al*., 2002; Stone *et al*., 2004). In contrast, the DGAT1 homologues in yeast, Are1 and Are2, serve primarily as sterol esterification enzymes (Yang *et al*., 1996) and play only a minor role in triacylglycerol synthesis (Oelkers *et al*., 2002; Stone *et al*., 2004).

Studies of microsomes from rat adipocytes or livers revealed that DGAT activity (DGAT1 and DGAT2) was present in the ER (Wilgram *et al*., 1963; Coleman *et al*., 1976; Andersson *et al*., 1994; Rusinol *et al*., 1994; Lehner *et al*., 1996). Studies using tobacco plant cells overexpressing tung tree DGAT1 and DGAT2 pinpointed them to distinct, non-overlapping regions of the ER, suggesting different subcellular localization of DGAT1 and DGAT2 (Shockey *et al*., 2006).

Recently, DGAT2, but not DGAT1 has been shown to associate with lipid droplets (Kuerschner *et al*, 2008; Stone *et al*., 2009). DGAT2 may be directly embedded on the surface of lipid droplets and makes them another site for triacylglycerol synthesis. Murine DGAT2 has a long hydrophobic region spanning amino acids 66-115 that comprises either a single extended transmembrane domain or two transmembrane domains (Stone *et al*., 2006). The former conformation could allow the enzyme to bind directly to the lipid droplet surface (Stone *et al*., 2009). Indeed, a fraction of DGAT2 overexpressed in COS-7 cells co-purified with lipid droplets and was not due to ER contamination (Kuerschner *et al*., 2008). Other studies have provided additional evidence for DGAT2 being localized to lipid droplets. DGAT2 in the fungus *Mortierella ramanniana* was purified from the lipid body fraction (Lardizabal *et al*., 2001), and Dga1 activity in yeast is enriched in lipid droplet fractions (Sorger *et al*., 2002). A more likely explanation is that DGAT2 is present in the ER bilayer and is in close proximity to the lipid droplets (Stone *et al*., 2009). Various proteomic analyses of isolated lipid droplets did not identify DGAT2 as a droplet component (Wu *et al*., 2000; Basaemle *et al*., 2004; Fujimoto *et al*., 2004; Liu *et al*., 2004; Umlauf *et al*., 2004; Beller *et al*., 2006; Cermelli *et al*., 2006; Sato *et al*., 2006; Turro *et al*., 2006; Wan *et al*., 2007). Despite the uncertainty of the exact localization of DGAT2, the association of DGAT2 with lipid droplets corroborates the dedicated role of DGAT2 in bulk triacylglycerol synthesis and storage, while the ER-resident DGAT1 may be more important for synthesis of triacylglycerols destined for secretion (Kuerschner *et al*., 2008). This is consistent with previous findings in McArdle RH7777 cells

showing that overexpression of DGAT1 did not lead to a significant increase in intracellular triacylglycerol level, while DGAT2 overexpression resulted in formation of large cytosolic lipid droplets (Stone *et al*., 2004).

The membrane topology of DGAT2 suggests that the active site of DGAT2 resides on the cytosolic side of the ER, therefore, contributes only to the overt (cytosolic) activity (Stone *et al*., 2006), which synthesizes triacylglycerols destined for deposition into cytosolic lipid droplets (Waterman *et al*., 2002). In contrast, DGAT1 accounts for both overt and latent (luminal) activities (Stone *et al*., 2006; Yen *et al*., 2008), the latter of which may contribute to synthesis of triacylglycerols for incorporation into lipoproteins and secretion into the circulation (Waterman *et al*., 2002). The topology of DGAT1 indicates that the active site of DGAT1 is present in the ER lumen (McFie *et al*., 2010), suggesting a role for DGAT1 in lipoprotein assembly and secretion. In support of this, overexpression of DGAT1 in rat hepatoma McArdle RH7777 cells stimulated VLDL secretion (Liang *et al*., 2004). Additionally, overexpression of DGAT1 in mice resulted in increased latent DGAT activity in liver microsomes, which in turn caused a corresponding increase in the rate of VLDL secretion (Yamazaki *et al*., 2005).

In addition to its association with the ER and lipid droplets, DGAT2 also interacts with mitochondria (Stone *et al*., 2009). Immunofluorescence studies demonstrated that DGAT2 co-localizes with mitochondria, and biochemical fractionation revealed the abundant presence of DGAT2 in mitochondria-associated membranes (MAM), specialized domains of the ER that co-isolate and physically interact with mitochondria. MAM is enriched in activities of lipid biosynthetic enzymes, such as ACAT, DGAT, and phosphatidylserine synthase-1 and -2 (Cui *et al*., 1993; Rusinol *et al*., 1994; Stone *et al*., 2009). A mitochondrial targeting signal was identified in murine DGAT2 between amino acids 61 and 66. It is presumed that instead of being present directly in mitochondrial membranes, DGAT2 may be present in the MAM compartment and interact with mitochondria via its mitochondrial targeting sequence. It is unlikely that DGAT2 is present at the mitochondria membrane as DGAT2 has not been found in the mitochondrial proteomes from human and mouse tissues (Mootha *et al*., 2003; Taylor *et al*., 2003; Johnson *et al*., 2007). Although DGAT1 does not associate with mitochondria, it is also present in MAM (Stone *et al*., unpublished observations), which sediments with mitochondria (at 10,000 x *g*) (Vance, 1989). Mitochondria and MAM together are referred to as the crude mitochondrial fraction.

The reason for the interaction of DGAT2 with mitochondria is not clear. This arrangement may promote the transfer of energy and exchange of substrates for triacylglycerol synthesis (Stone *et al*., 2009), because energy for activating fatty acids comes from mitochondria, and many enzymes that catalyze triacylglycerol formation are located in both mitochondria and ER (Coleman *et al*., 2000, 2004; Stone *et al*., 2009).

2.6 Physiological Functions of DGAT Enzymes

Our understanding of the physiological functions of DGAT enzymes in triacylglycerol metabolism stems largely from gene targeting studies in mice, which in turn reveal how the two DGAT enzymes serve fundamentally different roles in mammalian energy metabolism (Smith *et al*., 2000; Chen *et al*., 2002a; Stone *et al*., 2004). Both DGAT1 and DGAT2 are ubiquitously expressed, with the highest levels of expression found in tissues that are active in triacylglycerol synthesis, such as white adipose tissue, liver, small intestine, and mammary gland (Yu *et al*., 2004; Yen *et al*., 2008). Consequently, beyond their roles in systemic energy metabolism, DGAT enzymes also have been hypothesized to have a tissue-specific function in several physiologic processes in these tissues.

2.6.1 Functions of DGAT Enzymes in Lipid and Energy Metabolism *In vivo*

Targeted disruption of *Dgat1* produced mice that are viable and have less adipose mass (~50% reduction). DGAT1-deficient (*Dgat1*$^{-/-}$) mice are leaner than wild-type mice and are resistant to diet-induced obesity through a mechanism involving increased energy expenditure (Smith *et al*., 2000). Although the mechanism is not entirely clear, several factors have been suggested to play a role. *Dgat1*$^{-/-}$ mice exhibited increased physical activity (Smith *et al*., 2000; Chen *et al*., 2002a) and thermogenesis (Chen *et al*., 2003), the latter may be attributable to the increased expression of uncoupling protein 1, a major mediator of nonshivering thermogenesis. Furthermore, increased sensitivity to leptin, an adipocyte-derived hormone that enhances energy expenditure, was observed in *Dgat1*$^{-/-}$ mice (Chen *et al*., 2002a). Triacylglycerol levels in nonadipose tissues such as liver and skeletal muscle were moderately reduced in *Dgat1*$^{-/-}$ mice and plasma triacylglycerol levels were normal (Smith *et al*., 2000). *Dgat1*$^{-/-}$ mice also had improved glucose tolerance as they have increased insulin sensitivity (Chen *et al*., 2002a;

Chen *et al*., 2003). Adult *Dgat1*$^{-/-}$ mice appeared to have dry fur and hair loss, which may be associated with DGAT1's pleiotropic acyltransferase activities (Yen *et al*., 2008). Since wax diesters are a major species of fur lipids in rodents, the abolished wax diester synthase activity of DGAT1 may underlie the missing fur lipids in *Dgat1*$^{-/-}$ mice (Chen *et al*., 2002b). Certain retinyl esters have been shown to promote hair growth (Bazzano *et al*., 1993). Therefore, disrupted ARAT activity of DGAT1 may result in decreased production of retinyl esters, which in turn may lead to the abnormal development of hair in *Dgat1*$^{-/-}$ mice.

Unlike DGAT1, DGAT2 is essential for life. DGAT2-deficient (*Dgat2*$^{-/-}$) mice die in the early postnatal period, with newborn pups surviving only 6-8 hours after birth (Stone *et al*., 2004). At least two mechanisms contribute to their early mortality. One mechanism is the marked reduction in substrates for energy metabolism. The carcass triacylglycerol content of *Dgat2*$^{-/-}$ mice was reduced by ~90%, and triacylglycerols were nearly undetectable in *Dgat2*$^{-/-}$ livers. The plasma levels of triacylglycerols, free fatty acids, and glucose were reduced by 70-90%. *Dgat2*$^{-/-}$ mice also exhibited severe skin abnormalities with increased transepidermal water loss, which led to rapid dehydration and postnatal lethality. The skin abnormalities were attributable to the deficiency of an essential fatty acid, linoleic acid in *Dgat2*$^{-/-}$ mice. Linoleic acid is required for the synthesis of acylceramide, which is required for maintaining the permeability barrier of the skin.

2.6.2 Functions of DGAT Enzymes in Adipose Tissue

Adipose tissue is the predominant energy storage depot in the body, and DGAT enzymes are believed to play an important role in triacylglycerol synthesis for energy storage (Yu *et al*., 2004; Yen *et al*., 2008). High levels of DGAT activity have been found in isolated rat adipocytes (Coleman *et al*., 1976) and in differentiated 3T3-L1 adipocytes (Coleman *et al*., 1978; Cases *et al*., 1998; Cases *et al*., 2001).

In addition to its synthetic function for energy storage, DGAT1 may also attenuate the deleterious effects of excessive fatty acids and their metabolites in non-adipose tissues by incorporating them into innocuous triacylglycerols in adipose tissue. For example, overexpression of DGAT1 in white adipose tissue may protect against lipotoxicity in non-adipose tissues (Chen *et al*., 2002c). In response to a high-fat diet, aP2-*Dgat1* transgenic mice (C57BL/6 genetic background) overexpressing DGAT1 in white adipose tissue had increased

triacylglycerol deposition in white adipose tissue and became more obese than wild-type mice. However, triacylglycerol levels were normal in non-adipose tissues in association with normal glucose metabolism. In contrast, when fed a high-fat diet, no increased triacylglycerol content in white adipose tissue or obesity was observed in another line of aP2-*Dgat1* transgenic mice (FVB genetic background) overexpressing DGAT1. Consequently, these mice developed hyperglycemia, hyperinsulinemia, and hepatic steatosis in association with dramatically elevated plasma free fatty acid levels (Chen *et al*., 2005).

2.6.3 Functions of DGAT Enzymes in Lipoprotein Metabolism

2.6.3.1 Functions of DGAT Enzymes in the Intestine

Dietary triacylglycerols cannot be absorbed intact. In the intestine, the absorption of dietary triacylglycerols involves their initial hydrolysis in the intestinal lumen to free fatty acids and monoacylglycerol, which can cross from the gut to intestinal epithelial cells, subsequent re-synthesis in enterocytes, and final incorporation of newly synthesized triacylglycerols into nascent chylomicrons for secretion into the circulation (Yu *et al*., 2004). Although DGAT1 is highly expressed in the intestine, it appears to be non-essential for dietary triacylglycerol absorption and chylomicron assembly. *Dgat1*$^{-/-}$ enterocytes can still synthesize triacylglycerol-rich, chylomicron-sized particles (Buhman *et al*., 2002; Yu *et al*., 2004). However, post-absorptive plasma chylomicron levels were reduced in *Dgat1*$^{-/-}$ mice after a high-fat diet challenge, and lipid droplets accumulated in *Dgat1*$^{-/-}$ enterocytes when the mice were chronically fed a high fat diet. All these results suggest that *Dgat1*$^{-/-}$ mice process dietary triacylglycerols through enterocytes into circulation at a decreased but more sustained rate, but the precise role of DGAT1 in this process remains unknown.

In the intestine, DGAT2 expression was observed in mice, but was very low in humans (Cases *et al*., 2001). Because there are no adult *Dgat2*$^{-/-}$ mice (Stone *et al*., 2004), no data can be obtained regarding the role of DGAT2 in dietary triacylglycerol absorption in the intestine.

2.6.3.2 Functions of DGAT Enzymes in the Liver

In the liver, DGAT enzymes are actively involved in the production of triacylglycerols, which may be channeled into cytosolic lipid droplets or be incorporated into nascent

apolipoprotein-B (apoB)-containing, triacylglycerol-rich, very low density lipoproteins (VLDL). Therefore, DGAT enzymes are believed to have a significant impact on the assembly and secretion of VLDL. Overexpression of human DGAT1 in rat hepatoma McArdle RH7777 cells resulted in increased synthesis of triacylglycerols, decreased apoB degradation, and subsequently, enhanced secretion of triacylglycerols in the form of VLDL (Liang *et al*., 2004). In two separate *in vivo* studies, overexpression of DGAT1 in mouse liver via adenovirus increased cytosolic triacylglycerol levels in hepatocytes (Yamazaki *et al*., 2005; Millar *et al*., 2006). However, only one group observed an increase in triacylglycerol secretion (Yamazaki *et al*., 2005). In both studies, overexpression of DGAT2 increased hepatic triacylglycerol levels, without augmenting VLDL secretion (Yamazaki *et al*., 2005; Millar *et al*., 2006). However, knockdown of DGAT2 in the mouse liver with antisense oligonucleotide lowered the rate of hepatic triacylglycerol secretion and plasma triacylglycerol levels (Yu *et al*., 2005; Liu *et al*., 2008). Thus, the existing data suggest that DGAT1 has more of a role in VLDL production in the liver than DGAT2.

2.6.4 Functions of DGAT Enzymes in Mammary Gland

DGAT1 appears to be required for the development of the mammary gland in mice (Smith *et al*., 2000). Instead of producing milk low in fat, female *Dgat1*$^{-/-}$ mice failed to lactate completely. Neutral lipid-staining droplets were nearly absent in the apical regions of epithelial cells and in the ductal lumens of mammary glands from *Dgat1*$^{-/-}$ females. DGAT1 deficiency is believed to cause impaired mammary epithelial proliferation and functional differentiation, which in turn result in impaired mammary gland development (Cases *et al*., 2004).

2.7 Regulation of DGAT Enzymes

Although gene targeting studies have shed light on their metabolic functions, very little is known about the mechanisms responsible for regulating DGAT enzymes. Since DGAT enzymes function at a branch point in the glycerolipid biosynthetic pathway, one might expect these enzymes to be regulated as the diacylglycerol used by DGAT for triacylglycerol synthesis could potentially be used for phospholipid biosynthesis. Previous studies have demonstrated

that DGAT enzymes may divert the flux of diacylglycerol from phospholipid synthesis toward triacylglycerol synthesis (Bagnato *et al*., 2003).

In mice, DGAT2 mRNA expression appears to be much more sensitive to dietary conditions than DGAT1 (Meegalla *et al*., 2002). DGAT2 mRNA levels were reduced in white adipose tissue and liver upon a 24 hour fast, and increased with refeeding. In contrast, DGAT1 did not exhibit such a pattern of change of mRNA levels. These findings suggest that DGAT1 might instead be regulated by post-transcriptional mechanisms rather than by changes in gene expression. In addition, in both 3T3-L1 fibroblasts and adipocytes, overexpression of DGAT1 by a recombinant adenovirus construct led to a 20-fold increase in DGAT1 mRNA levels (Yu *et al*., 2002a). However, nearly no increase in DGAT activity was observed in fibroblasts and only a 2 fold increase was present in adipocytes, corroborating the possibility of extensive posttranscriptional regulation of DGAT1.

Early studies in rat hepatocytes and white adipose tissue have provided evidence indicating that *in vitro* DGAT activity could be regulated post-transcriptionally via a phosphorylation-dephosphorylation mechanism. DGAT activity appeared to be lower in rat liver microsomes isolated in the presence of 50 mM fluoride, a phosphatase inhibitor, than in control microsomes isolated without fluoride (Haagsman *et al*., 1981). Furthermore, DGAT activity in both rat hepatocytes and white adipose tissue could be inactivated by a cytosolic factor in the presence of ATP and magnesium, conditions promoting protein phosphorylation (Haagsman *et al*., 1982; Rodriguez *et al*., 1992). The inactivated DGAT could be reactivated by incubation with partially purified protein phosphatase(s) from rat liver, and inactivated again with further addition of ATP and magnesium in the presence of the cytosolic fraction. These results suggest the existence of a protein kinase responsible for regulating DGAT activity. Partial purification of an inhibitory factor from adipose tissue suggested that it could be a tyrosine kinase, since it was inhibited by tyrosine kinase inhibitors and was insensitive to serine/threonine kinase inhibitors (Lau *et al*., 1996). Both human and mouse DGAT1, but not DGAT2, contain a single conserved sequence for tyrosine phosphorylation (Oelkers *et al*., 1998; Cases *et al*., 2001). However, when this site was mutated in human DGAT1 and expressed in 3T3-L1 adipocytes, no significant effect on *in vitro* DGAT activity or overall cellular triacylglycerol accumulation was observed (Yu et al., 2002a). Therefore, it appears unlikely that tyrosine phosphorylation at this site regulates DGAT1 activity. However, the authors of

this study did not determine if the DGAT activity of the tyrosine mutant could still be inhibited with cytosol in the presence of ATP and magnesium.

There has been some confusion with respect to regulation of DGAT activity by phosphorylation as different laboratories have published conflicting results regarding regulation by 3'-5'-cyclic adenosine monophosphate (cAMP). Incubation of isolated rat hepatocytes with cAMP decreased their DGAT activity, suggesting regulation by cAMP-dependent pathways involving PKA (Haagsman *et al*., 1981; Lehner *et al*., 1996). However, in another study in rat adipose tissue, the inhibitory effect of cAMP on DGAT activity was not observed, indicating that some other kinases other than PKA may be involved in the regulation of DGAT (Rodriguez *et al*., 1992; Lehner *et al*., 1996). More interestingly, stimulatory effect of cAMP on DGAT activity was observed in hamster fibroblasts incubated with cAMP (Mazière *et al*., 1986; Lehner *et al*., 1996). Most of these earlier studies are difficult to interpret since we now know there are two DGAT enzymes, both of which contain several putative phosphorylation sites, and therefore, these previous studies would not distinguish which DGAT was particularly modified in particular experiments. DGAT1, but not DGAT2, has the consensus sequence for two PKA phosphorylation sites (T15 and S244), suggesting that DGAT1 could be subject to regulation by cAMP-dependent pathways (Figure 2.4).

2.8 Preliminary Data

All preliminary experiments were performed by my supervisor, Dr. Scot Stone in the laboratory of Dr. R.V. Farese (Gladstone Institute of Cardiovascular Disease, San Francisco, CA).

2.8.1 *In vitro* Phosphorylation of DGAT1

To determine if DGAT1 and/or DGAT2 were subject to phosphorylation, rat hepatoma McArdle RH7777 cells overexpressing FLAG-tagged DGAT1 or DGAT2 were radiolabeled with [^{32}P]inorganic phosphate. After immunoprecipitation of DGAT1 and DGAT2 from the cells, phosphorylation was assessed by SDS-PAGE and autoradiography. Only DGAT1, and not DGAT2, appeared to be phosphorylated (Figure 2.5). Several bands labeled with [^{32}P] inorganic phosphate were observed that corresponded to DGAT1, suggesting that DGAT1 may

have multiple phosphorylation sites. The effect of phosphorylation on DGAT1 activity was then determined. Microsomes and cytosol were prepared from McArdle RH7777 cells overexpressing DGAT1. *In vitro* DGAT activity in microsomes was markedly inhibited by a cytosolic factor in the presence of magnesium and ATP (Figure 2.6). When incubated with alkaline phosphatase, DGAT1 activity was increased ~5-fold, compared to the untreated control sample (Figure 2.7).

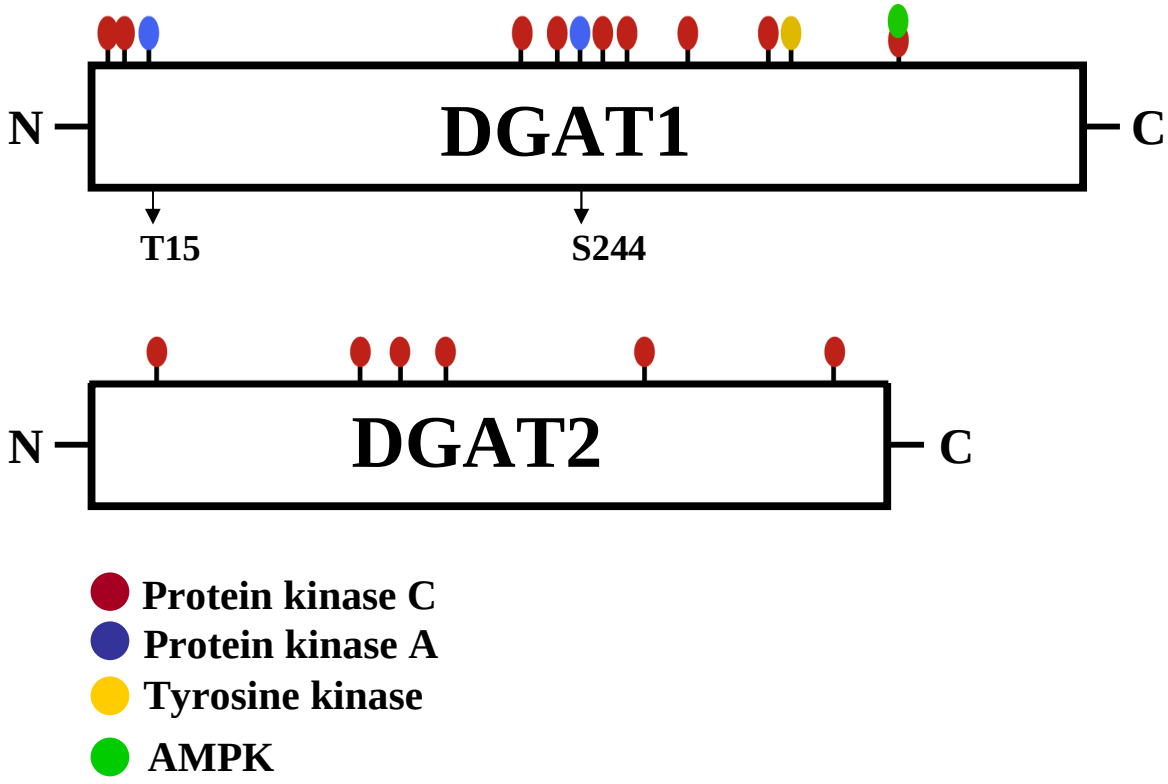


Figure 2.4 Predicted Phosphorylation Sites of DGAT Enzymes for Various Kinases.
DGAT1, but not DGAT2, has the consensus pattern [R/K](2)-X-[S/T] for PKA phosphorylation site at threonine-15 and serine-244.

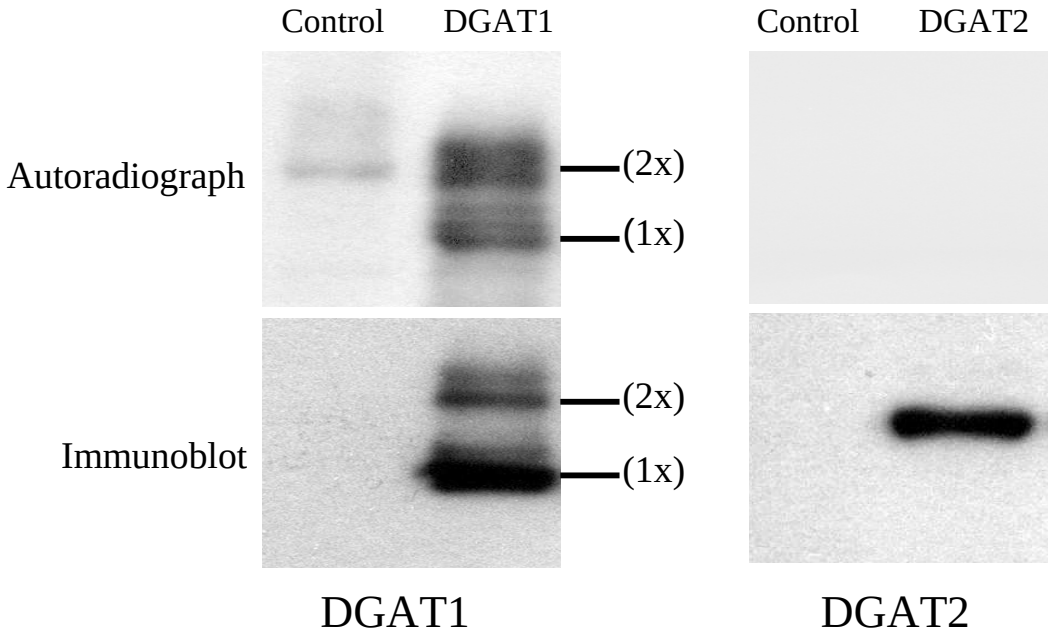


Figure 2.5 DGAT1, but not DGAT2 is Phosphorylated.
McArdle RH7777 cells overexpressing FLAG-tagged DGAT1 or DGAT2 were incubated with 0.5 mCi [^{32}Pi] for 4h. DGAT1 and DGAT2 were then immunoprecipitated, separated by SDS PAGE and transferred to polyvinylidene difluoride membranes. Membranes were exposed to film to detect phosphorylation, followed by immunoblotting for DGAT1 and DGAT2 using a monoclonal anti-FLAG antibody. McArdle RH7777 cells transfected with the expression vector lacking an insert were used as a control. 1x represents the monomer of DGAT1, 2x represents the dimer of DGAT1. This experiment was carried out by Dr. Scot Stone.

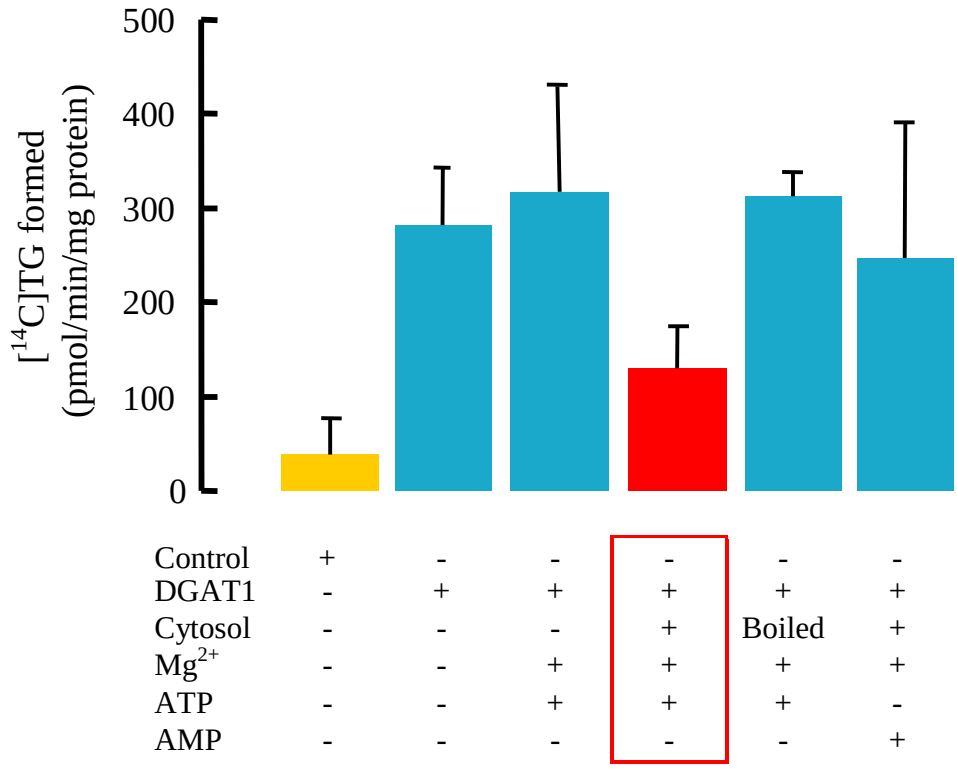


Figure 2.6 Inactivation of DGAT1 under Conditions Promoting Protein Phosphorylation. Microsomes and cytosol were prepared from McArdle RH7777 cells overexpressing DGAT1. Microsomes were then pre-incubated under conditions outlined above, and DGAT activity was determined. McArdle RH7777 cells transfected with the expression vector lacking an insert were used as a control. TG: triacylglycerol. This experiment was carried out by Dr. Scot Stone.

2.8.2 *In vitro* Phosphorylation of DGAT1 by PKA

Using a candidate kinase approach, PKA was identified as one possible kinase that could regulate DGAT1. Partially purified DGAT1 was incubated with Mg^{2+}, [γ^{32}P]ATP, and either PKA, AMP-activated protein kinase (AMPK) or Casein kinase 2. Phosphorylation was detected by SDS-PAGE and autoradiography. A protein corresponding to DGAT1 was directly phosphorylated by PKA, and phosphorylation was prevented by a PKA inhibitor peptide, PKI (Figure 2.8). AMPK and CK2 did not phosphorylate DGAT1. *In vitro* DGAT activity in microsomes from McArdle RH7777 cells overexpressing DGAT1 was inhibited by PKA (Figure 2.9). Furthermore, treatment of intact COS-7 cells with either forskolin or dibutyryl-cAMP, two known PKA activators, inhibited *in vitro* DGAT activity by ~50% (Figure 2.10).

This preliminary data strongly supports our hypothesis that DGAT1, and not DGAT2, is subject to regulation by phosphorylation/dephosphorylation and that PKA-dependant pathways are likely involved.

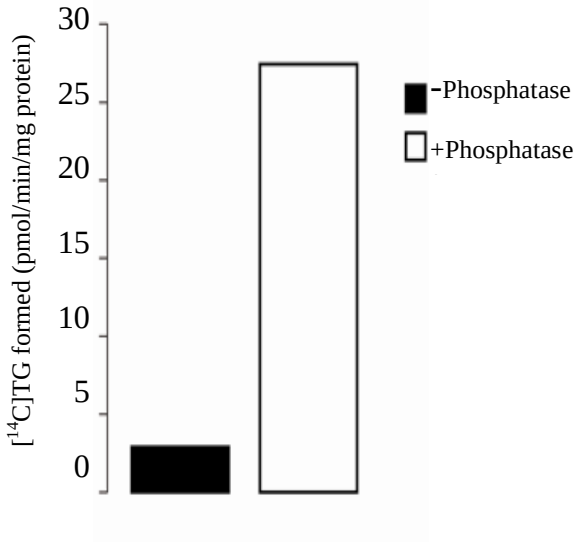


Figure 2.7 Stimulation of DGAT1 under Conditions Promoting Protein Dephosphorylation.
Microsomes were prepared from McArdle RH7777 cells overexpressing DGAT1. Microsomes were then pre-incubated with or without alkaline phosphatase, and DGAT activity was determined. TG: triacylglycerol. This experiment was carried out by Dr. Scot Stone.

2.9 Rationale and Objectives of the Proposed Research

What is the biological significance of inhibiting DGAT1 activity by phosphorylation by PKA? I hypothesize that triacylglycerol synthesis and breakdown are coordinately regulated processes. The role of PKA-dependent lipolytic pathways has been well studied. In the fasted state, β-adrenergic stimulation of adipocytes activates adenylyl cyclase, thereby producing cAMP that activates PKA. PKA phosphorylation of hormone sensitive lipase (HSL) and perilipin leads to subsequent activation and translocation of HSL to the lipid droplet, which in turn promotes hydrolysis of triacylglycerols (Souza *et al*., 2002; Sztalryd *et al*., 2003; Tansey *et al*., 2003; Zhang *et al*., 2003). Additionally, PKA phosphorylation of perilipin activates adipose triacylglycerol lipase (ATGL), which is mediated through CGI-58, a protein activator of ATGL (Lass *et al*., 2006) that interacts with perilipin (Yamaguchi *et al*., 2004; Subramanian *et al*., 2004). It was reported that phosphorylation of perilipin resulted in the dissociation of CGI-58 from perilipin, which in turn allowed CGI-58 to interact with ATGL and activate its lipase activity (Granneman *et al*., 2009).

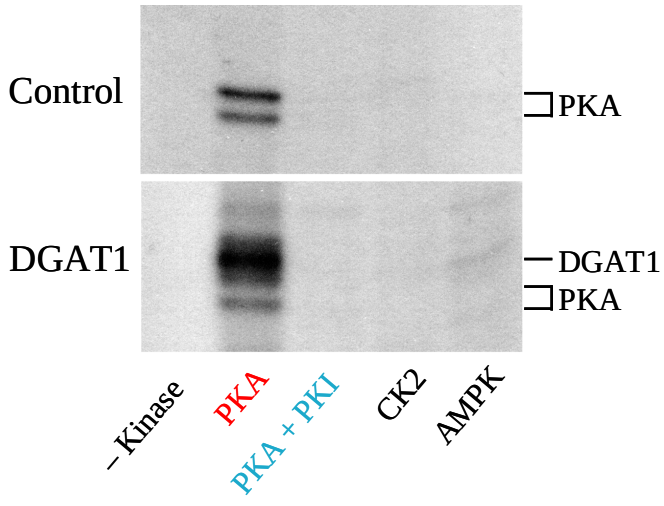


Figure 2.8 *In vitro* Phosphorylation of DGAT1 by PKA.
DGAT1 was partially purified from McArdle RH7777 cells overexpressing DGAT1 and incubated with Mg^{2+}, [γ^{32}P]ATP, and either PKA, casein kinase 2 (CK2) or AMP-activated protein kinase (AMPK). Samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and exposed to film to detect phosphorylation. PKI is a PKA inhibitor. PKA underwent auto-phosphorylation. McArdle RH7777 cells transfected with the expression vector lacking an insert were used as a control. This experiment was carried out by Dr. Scot Stone.

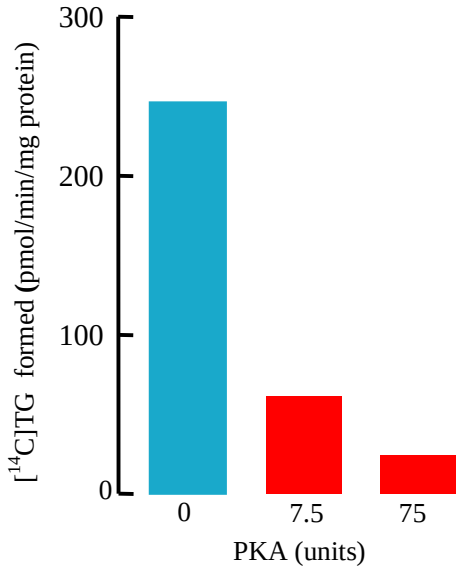


Figure 2.9 Inhibition of *In vitro* DGAT Activity by PKA.
Microsomes were isolated from McArdle RH7777 cells overexpressing DGAT1 and pre-incubated with various units of PKA at room temperature for 30 min. I*n vitro* DGAT activity was then determined. TG: triacylglycerol. This experiment was carried out by Dr. Scot Stone.

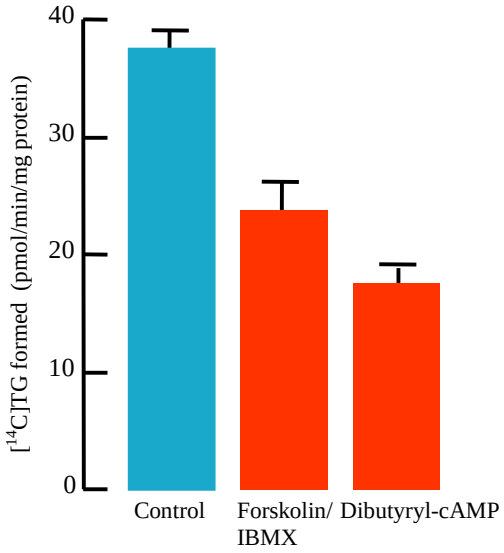


Figure 2.10 Inhibition of Endogenous DGAT Activity by PKA Activators.
COS-7 cells were incubated with either 100 µM forskolin and 100 µM IBMX or 0.5 mM dibutyryl-cAMP for 20 min. *In vitro* DGAT activity was then measured in cell lysates. IBMX: isobutylmethylxanthine; TG: triacylglycerol. This experiment was carried out by Dr. Scot Stone.

However, to our knowledge, what happens to the pathways of triacylglycerol synthesis in adipose tissue during lipolysis is not clear. We hypothesize that during lipolysis, the rate of triacylglycerol synthesis in adipose tissue is decreased in order to promote the efficient net release of stored energy. Under fasting conditions, phosphorylation of DGAT1 by PKA would inhibit its enzymatic activity, decreasing triacylglycerol synthesis. In contrast, during the fed state, DGAT1 would be in a dephosphorylated and active form, promoting net triacylglycerol synthesis and storage. I shall determine the significance of PKA phosphorylation in regulating DGAT1 and triacylglycerol synthesis.

The specific objectives of this thesis were:

1. Determining DGAT activity during lipolysis in 3T3-L1 adipocytes.
2. Mapping PKA phosphorylation site(s) of DGAT1 and studying their functional importance.

3.0 MATERIALS AND METHODS

3.1 Reagents

Names of reagents and their suppliers are listed in Table 3.1. Addresses of reagent suppliers are listed in Table 3.2. Names of primer pairs used for site-directed mutagenesis of DGAT1 and their sequences are listed in Table 3.3.

Table 3.1: List of Reagents and Suppliers

General Reagent	**Supplier**
Anti-FLAG M2-Agarose Beads	Sigma-Aldrich
Bovine Serum Albumin-essentially fatty acid free	Sigma-Aldrich
Brilliant Blue R	Sigma-Aldrich
[^{14}C]Oleoyl-CoA	GE Healthcare
Chlorophenylthio-Cyclic AMP	Sigma-Aldrich
Cycloheximide	Sigma-Aldrich
Diethyl-p-Nitrophenyl Phosphate	Sigma-Aldrich
1,2-Dioleoyl-sn-Glycerol	Sigma-Aldrich
Disuccinimidyl Suberate	Pierce
3X FLAG Peptide	Sigma-Aldrich
[^{3}H]Glycerol (1mCi/mL)	American Radiolabeled Chemicals, Inc.
Polyethylenimine	Polysciences
Protease Inhibitor Tablet (Complete, Mini)	Roche
Protein Kinase A (catalytic subunit)	Promega
Oleic Acid	Sigma-Aldrich
Salmon Sperm DNA	Sigma-Aldrich
Bacterial Culture Reagent	**Supplier**
Ampicillin	Bio Basic Inc.
Tryptone	DIFCO
Yeast Extract	EMD

NZ-Amine A® from Bovine Milk	Sigma-Aldrich
Mammalian Cell Culture Reagent	**Supplier**
Antibiotic-Antimyotic (100X)	Invitrogen
Dulbecco's Modified Eagle's Medium – High Glucose	Lonza
Dexamethasone	Sigma-Aldrich
Fetal Bovine Serum (Canadian Origin)	PAA Laboratories Inc.
Horse Serum	Invitrogen
Isobutylmethylxanthine	Sigma-Aldrich
Insulin (10mg/mL)	Sigma-Aldrich
Trypsin (0.05 %,1x, EDTA)	Invitrogen
Yeast Culture Reagent	**Supplier**
Galactose	Acros Organics
Raffinose	Sigma-Aldrich
Yeast Nitrogen Base	DIFCO

Table 3.2 List of Names and Addresses of Reagent Suppliers

Supplier	Address
Acros Organics	Morris Plains, New Jersey, USA
American Radiolabeled Chemicals, Inc.	St. Louis, Missouri, USA
Bio Basic Inc.	Markham, Ontario, Canada
Bio-Rad	Hercules, California, USA
Denville Scientific	St. Laurent, Quebec, Canada
DIFCO	Sparks, Michigan, USA
EMD	Madison, Wisconsin, USA
GE Healthcare	Baie d'Urfe, Quebec, Canada
Invitrogen	Burlington, Ontario, Canada
Lonza	Walkersville, Maryland, USA
Matrix Science	London, UK
PAA Laboratories Inc.	Etobicoke, Ontario, Canada
Pierce	Rockford, Illinois, USA

Polysciences	Warrington, Pennsylvania, USA
Promega	Madison, Wisconsin, USA
Roche	Mississauga, Ontario, Canada
Sigma-Aldrich	Oakville, Ontario, Canada
Stratagene	La Jolla, California, USA
Whatman	Piscataway, New Jersey, USA
VWR	Mississauga, Ontario, Canada

Table 3.3: List of Primers (Invitrogen) for Site-directed Mutagenesis of DGAT1 (modifications are underlined)

Primer Name	Primer Sequence
S17A-F	5'-AGGACCGGCGCACGGGTTTCCGTCC-3'
S17A-R	5'-GGACGGAAACCCGTGCGCCGGTCCT-3'
S25A-F	5'-CGTCCAGGGTGGTGCTGGGCCCAAGGTAGAAG-3'
S25A-R	5'-CTTCTACCTTGGGCCCAGCACCACCCTGGACG-3'
S17/20/25A-F	5'-CGCACGGGTTGCCGTCCAGGGTGGTGCTGGGCCCAAGG-3'
S17/20/25A-R	5'-CCTTGGGCCCAGCACCACCCTGGACGGCAACCCGTGCG-3'
T15D-F	5'-CGGCGTCGGAGGGACGGCTCGCGGGTTTCC-3'
T15D-R	5'-GGAAACCCGCGAGCCGTCCCTCCGACGCCG-3'
S17D-F	5'-CGTCGGAGGACCGGCGACCGGGTTTCCGTCCAG-3'
S17D-R	5'-CTGGACGGAAACCCGGTCGCCGGTCCTCCGACG-3'
S20D-F	5'-GGCTCGCGGGTTGACGTCCAGGGTGGTAGT-3'
S20D-R	5'-ACTACCACCCTGGACGTCAACCCGCGAGCC-3'
S25D-F	5'-CGTCCAGGGTGGTGATGGGCCCAAGGTAGAAG-3'
S25D-R	5'-CTTCTACCTTGGGCCCATCACCACCCTGGACG-3'
S17/20/25D-F	5'-ACCGGCGACCGGGTTGACGTCCAGGGTGGTGATGGGCCC-3'
S17/20/25D-R	5'-GGGCCCATCACCACCCTGGACGTCAACCCGGTCGCCGGT-3'
S17/20/25A-F (yeast)	5'-GACCGGCGCGCGGGTTGCCGTCCAGGGTGGTGCTGGGCCCA-3'
S17/20/25A-R (yeast)	5'-TGGGCCCAGCACCACCCTGGACGGCAACCCGCGCGCCGGTC-3'

3.2 Bacteria Strain, Yeast Strain and Media Preparations

E. coli DH5α competent cells (Invitrogen) were used as the host to propagate plasmids. XL10-Gold Ultracompetent *E. coli* cells (Stratagene) were used as the host to recover very low amounts of mutated plasmids during site-directed mutagenesis.

Luria-Bertani (LB) and Terrific Broth (TB) liquid media were used to propagate bacterial cells. LB was prepared by combining 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl in 1 L of double distilled water. TB was prepared by combining 12 g of tryptone, 24 g of yeast extract and 4 mL of glycerol in 900 mL of double distilled water. Both solutions were sterilized by autoclaving for 20 min at 15 lb/sq. in. After autoclaving, 100 mL of a sterile solution containing 0.17 M KH_2PO_4 and 0.72 M of K_2HPO_4 was added to TB. Ampicillin was added to the medium to a final concentration of 50 µg/mL when required for the selection and propagation of bacteria with a vector containing an Ampicillin-resistant gene. LB agar plates were made by adding 15 g of agar to 1 L of LB medium. After autoclaving, the medium was cooled and mixed with ampicillin (final concentration of 50 µg/mL) and poured into petri dishes. The LB agar plates were stored at 4 ºC until used.

NZY^+ broth used for the transformation of XL10-Gold ultracompetent cells was prepared by combining 10 g of NZ-amine, 5 g of yeast extract, and 5 g NaCl in 1 L of deionized water. The solution was adjusted to pH 7.5 and sterilized by autoclaving as described above. Prior to use, NZY^+ broth was supplemented with filter-sterilized 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$ and 0.004% (w/v) glucose.

Yeast Strain *Saccharomyces cerevisiae* H1246MATα (Sandager *et al*., 2002) was used to express murine DGAT1 and various mutants.

Synthetic complete medium lacking uracil (SC-Ura medium) was used to select and propagate yeast cells with a vector containing a gene for uracil synthesis. SC-Ura medium was prepared by dissolving 6.7 g of yeast nitrogen base, 0.1 g of each of adenine, arginine, cysteine, leucine, lysine, threonine and tryptophan, 0.05 g of each of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine in 800 mL of deionized water. This mixture was autoclaved as described above. Once cooled, 200 mL of filter-sterilized 10% raffinose was added to complete the SC-Ura medium. SC-Ura agar plates were made by adding 20 g of agar to 1 L of SC-Ura medium. After autoclaving, the medium was cooled and poured into petri dishes. The SC-Ura agar plates were stored at 4 ºC until used.

SC-Ura induction medium was used to induce the expression of wild-type DGAT1 and various mutants downstream of the *GAL1* promoter inducible by galactose. It was prepared as the SC-Ura medium with the exception that 100 ml of filter-sterilized 20% galactose and 100 ml of filter-sterilized 10% raffinose were added to the 800 mL mixture to complete the medium.

3.3 Mammalian Cell Culture

3T3-L1 cells and McArdle RH7777 rat hepatoma cells stably expressing FLAG-tagged DGAT1 (Stone *et al*., 2004) were kindly provided by Dr. R.V. Farese (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). Human embryonic kidney 293T (HEK293T) cells were gifts from Dr. W. J. Roesler (University of Saskatchewan, Saskatoon, SK). All cells were maintained at 37 ºC with 5% CO_2. McArdle RH 7777 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 10% horse serum. 3T3-L1 cells and HEK293T cells were maintained in DMEM with 10% FBS. 3T3-L1 cells were induced to differentiate into adipocytes by incubating ~90% confluent pre-adipocytes in DMEM supplemented with 10% FBS, 10^{-5} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 μg/mL insulin for 72 hr (Brasaemle *et al*., 1997). Medium was changed every 24 hr. 72 hr post-induction, the differentiation medium was withdrawn and the cells were maintained in DMEM containing 10% FBS.

3.4 Immunoaffinity Purification of DGAT1

The protocol described refers to cells propagated in a 100 mm tissue culture plate. All manipulations were performed at 4 °C.

3.4.1 Subcellular Fractionation

McArdle RH7777 cells stably expressing FLAG-tagged DGAT1 were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by scraping, and collected by centrifugation at 1000 × *g* for 2 min. The cell pellet was resuspended in 500 μL of ice-cold PBS containing 50 mM of phosphatase inhibitor, NaF and protease inhibitors. Cells were lysed by 15 passages through a 27-gauge needle. Cell debris and nuclei were pelleted by

centrifugation at 1000 × *g* for 2 min. The supernatant was centrifuged at 16,000 × *g* for 10 min to pellet crude mitochondrial fraction that contains mitochondria and MAM (Stone *et al*., 2009). The 16,000 × *g* supernatant was subjected to centrifugation at 100,000 × *g* for 30 min to pellet microsomes. Crude mitochondrial fraction and microsomes were combined and solubilized in 400 µL of PBS containing 0.5% SDS and 0.5% Triton X-100 in the presence of phosphatase and protease inhibitors, which is referred to as the solubilization buffer from now on.

3.4.2 Immunoprecipitation of DGAT1

40 µL of anti-FLAG M2-agarose beads were washed twice with ice-cold PBS and incubated with the solubilized crude mitochondrial and microsomal fractions prepared from above at 4 °C for 2 hr (rotating). Agarose beads were pelleted by centrifugation at 16,000 x *g* for 1 min, and washed 5 times with the solubilization buffer. FLAG-tagged DGAT1 was eluted by incubating agarose beads with 30 µL of the solubilization buffer containing 3X FLAG peptide (final concentration of 0.5 µg/µL) at 4°C for 30 min (rotating). Agarose beads were pelleted by centrifugation at 16,000 x *g* for 1 min and the supernatant containing partially purified DGAT1 was removed.

3.4.3 Western Blot Analysis

Immunoprecipitated samples (30 µL) were incubated with 5X SDS loading buffer (7.5 µL) (250 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 1% bromophenol blue, and 25% β mercaptoethanol) at 37 ºC for 20 min, followed by separation by SDS-PAGE on a 10% polyacrylamide gel in the running buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1 % (w/v) SDS) at 160 volts for 1 hr. Samples were incubated at 37 ºC because DGAT1 protein is very hydrophobic (Stone *et al*., 2006) and forms aggregates at higher temperatures. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) at 350 mA for 2 hr in the transfer buffer consisting of 62.5 mM boric acid (pH 8.0). The membrane was subsequently blocked in 5% milk at room temperature (RT) for 1 hr and probed overnight at 4 °C with a mouse monoclonal anti-FLAG-M2 antibody (Sigma-Aldrich, 1:5,000). The membrane was then incubated with a goat-anti-mouse polyclonal antibody conjugated to horseradish peroxidase (Bio-Rad, 1:8,000) for 1 hr at RT. The membrane bound antibody-protein complexes were detected using SuperSignal West Pico Chemiluminescent Substrate

(Pierce) as described by the manufacturer and exposed to Hyblot Cl film (Denville Scientific).

3.5 MS Analysis of DGAT1

In order to obtain sufficient quantities of DGAT1 for MS analysis, DGAT1 was immunoprecipitated from ten 100 mm culture dishes of McArdle RH7777 cells stably expressing FLAG-tagged DGAT1 as described above. Procedures were scaled up accordingly. The immunoprecipitates containing affinity purified DGAT1 were incubated with 100 units of the catalytic subunit of protein kinase A (PKA) in a final volume of 50 µL kinase buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 2.5 mM ATP, 5 mM $MgCl_2$, 50 mM NaF) at 30 °C for 30 min. Following separation by SDS-PAGE, proteins were visualized by staining with a solution containing 0.2% (w/v) Brilliant blue R, 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min, followed by destaining with 40% (v/v) methanol and 10% (v/v) acetic acid overnight. The protein band corresponding to DGAT1 was excised from the gel. PKA phosphorylation sites were then identified by analyzing multiple trypsin digests of affinity purified DGAT1 with high-performance liquid chromatography (HPLC)–electrospray ionization (ESI)-MS/MS, which is carried out by the MS facility of the NRC Plant Biotechnology Institute (Saskatoon, SK) on a fee-for-service basis.

3.6 Site-directed Mutagenesis

N-terminal FLAG-tagged murine DGAT1 in the eukaryotic expression vector pCDNA3.1 (Stone *et al*., 2004), or the cDNA encoding N-terminal FLAG-tagged DGAT1 cloned into a yeast expression vector pYES2 (Invitrogen) (generated by Pam McFie and Greg Lutz in our laboratory), were used as templates for mutagenesis reactions. The various mutations were introduced using the primer pairs listed in Table 3.3 and the QuikChange II XL site-directed mutagenesis kit (Stratagene). The PCR mixture was set up as follows (in a total reaction volume of 50 µL): 50 ng of DNA, 5 µL of 10X reaction buffer, 0.2 mM dNTPs, 125 ng each of forward and reverse primers, 5 µL of dimethyl sulfoxide (DMSO), and 1 µL of *PfuUltra* High Fidelity DNA polymerase. The thermal cycling parameters used for the PCR were as follows: initial denaturation at 95°C for 1 min, followed by 18 cycles at 95 °C for 50 sec, 60 °C

for 50 sec, and 68 °C for 6.5 min, with termination at 68 °C for 7 min. Following PCR, the product was treated with *Dpn*I endonuclease, and transformed into XL10-Gold ultracompetent cells as described by the manufacturer. Plasmid DNA was isolated from transformants as described by Sambrook *et al*. (1989). All constructs were sequenced to verify the presence of the intended mutations by the sequencing facility of the NRC Plant Biotechnology Institute (Saskatoon, SK).

3.7 Expression of DGAT1

3.7.1 Mammalian Cell Transfection

For each transfection, 60 µL of 0.1% polyethylenimine was gently mixed with a mixture containing 20 µg of plasmid DNA and 430 µL of 150 mM NaCl in a 14 mL polypropylene tube at RT for 10 sec. The transfection mixture was then incubated at RT for 10 min and added drop-wise to a 100 mm tissue culture plate containing HEK293T cells at 70% confluency. Cells were incubated at 37°C with 5% CO_2 for 4 hr, and then re-fed with fresh culture medium. 48 hr post-transfection, cells were harvested and used for experiments.

3.7.2 Yeast Transformation

For each transformation, 1 µg of plasmid DNA was added into a 14 mL polypropylene tube containing 100 µg of denatured salmon sperm DNA and 100 µL of competent yeast containing approximately 10^6 cells. 600 µL of PEG/LiAc solution (10 mM Tris-HCl, pH 7.5, 40% PEG-3350 solution, 100 mM lithium acetate, 1 mM EDTA,) was added to the mixture, followed by brief vortexing. The transformation mixture was then incubated at 30°C for 30 min with shaking at 250 rpm, and 70 µL of DMSO was added after the incubation. Yeast cells were heat shocked at 42°C for 15 min and transferred to a 1.5 mL microcentrifuge tube. Cells were collected by centrifugation at 16,000 x *g* for 15 sec. The pellet was resuspended in 500 µL of 1X TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), and plated on SC-Ura agar plates to select transformants with a vector containing a gene for uracil synthesis. A single colony was inoculated into 15 mL of SC-Ura medium and grown at 30 °C overnight. The overnight culture was diluted to an OD_{600} of 0.4 in 50 mL of SC-Ura induction medium and grown at

30 °C for 24 hr. After induction, yeast were harvested and used for experiments.

3.8 Measurement of *In vitro* DGAT Activity

3.8.1 Extraction of Total Cellular Membranes

All manipulations were performed at 4 °C.

The protocol described refers to mammalian cells propagated in a 100 mm tissue culture plate. Cells were washed twice with ice-cold PBS, harvested by scraping, and collected by centrifugation at 1000 x *g* for 2 min. The cell pellet was resuspended in 500 µL of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, and protease inhibitors), and passed through a 27-gauge needle 15 times to lyse cells. Cell debris and nuclei were removed by centrifugation at 1000 x *g* for 2 min. The supernatant was subjected to centrifugation at 100,000 x *g* for 30 min to pellet total cellular membranes. The membrane pellet was resuspended in 200 µL of ice-cold lysis buffer. In most experiments, 50 mM NaF was included in the lysis buffer to minimize dephosphorylation of DGAT1. In some experiments, cells were pre-incubated with 0.5 mM chlorophenylthio-3'-5'-cyclic adenosine monophosphate (CPT-cAMP) for 0-120 min before harvesting, to activate lipolysis.

The protocol described refers to yeast cells propagated in 10 mL of induction medium. 24 hr post-induction, yeast cells were collected by centrifugation at 1500 x *g* for 5 min. The cell pellet was resuspended in 500 µL of sterile water and transferred to a microcentrifuge tube. Yeast were collected by centrifugation at 16,000 x *g* for 30 sec and resuspended in 500 µL of ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol, 1 mM PMSF). Yeast were then lysed by adding an equal volume of acid-washed glass beads (425-600 µm size, Sigma-Aldrich), and bead beating the mixture 4 times for 1 min in a bead beater (VWR), with 1 min on ice in between beatings. Cell debri, nuclei, and the beads were pelleted by centrifugation at 1000 x *g* for 5 min. The supernatant was centrifuged at 100,000 x *g* for 30 min to pellet total cellular membranes. The membrane pellet was resuspended in 200 µL of ice-cold 50mM Tris-HCl (pH 7.4) buffer containing 250 mM sucrose, 50 mM NaF and protease inhibitors.

3.8.2 Analysis of Protein Expression Levels

The protein concentration of total cellular membrane extractions was determined using the Bio-Rad Protein Assay kit (Bio-Rad) as indicated by the manufacturer. Protein samples were adjusted to a final concentration of 1 µg/µL. Aliquots of protein samples were then subjected to western blot analysis as described in section 3.4.3.

3.8.3 *In vitro* DGAT Activity Assay

DGAT activity assays were carried out in 16 x 100 mm glass test tubes in a total volume of 200 µL. In brief, these assays measured the incorporation of radiolabelled oleoyl-CoA into triacyglycerol in a 10 min assay using sn-1, 2-diacylglcyerol and [^{14}C]oleoyl-CoA as substrates. A typical reaction contained 50-200 µg of protein in 100 µL of total cellular membrane extractions, which was added to 100 µL of assay mixture (200 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 25 mg/mL bovine serum albumin (BSA)-essentially fatty acid free, 400 mM 1,2-dioleoyl-sn-glycerol [in acetone, vehicle concentration 10% (v/v)], and 50 µM [^{14}C]oleoyl-CoA at 18 µCi/µMol). The reaction mixture was incubated at 37 ºC for 10 min, followed by termination of the reaction with 4 mL of chloroform: methanol (2:1, v/v). 800 µL of distilled water was then added, and the mixture was vortexed and incubated at RT for 1 hr. After incubation, the mixture was centrifuged at 1000 x *g* for 5 min to separate the organic and aqueous phases. The aqueous phase was removed by aspiration and the organic phase containing lipids was dried under air. Dry lipids were resuspended in 75 µL of chloroform: methanol (2:1, v/v) and applied to a 20 x 20 cm Thin Layer Chromatography (TLC) plate (Whatman), which was developed in hexane: diethyl ether: acetic acid (80: 20: 1, v/v/v). *In vitro* DGAT activity was determined by measuring the incorporation of radiolabelled [^{14}C] oleoyl-CoA into triacylglycerol. Triacylglycerol was visualized by staining lipids with iodine vapor. The band corresponding to triacylglycerol was scraped off the plate, and quantified by scintillation counting as described by Taylor *et al*. (1991).

In some experiments, *in vitro* DGAT activity assays were carried out with various concentrations 1,2-dioleoyl-sn-glycerol to establish a dioleoylglycerol substrate saturation curve of DGAT1.

3.9 Quantification of Triacylglycerol Synthesis

48 hr post-transfection, HEK293T cells transiently expressing wild-type DGAT1 and various mutants were washed twice with PBS, and incubated for 6 hr with fresh culture medium containing 10 μCi [^{3}H]glycerol and 0.375mM oleic acid complexed to 0.5% BSA. Cells were then washed twice with ice-cold PBS, harvested by scraping, and collected by centrifugation at 1000 x *g* for 2 min. Cells were resuspended in 500 μL of ice-cold PBS and disrupted by 15 passages through a 27-gauge needle. Cell debris and nuclei were pelleted by centrifugation at 1000 x *g* for 2 min. Lipids were extracted from 200 μL of the total cell lysate, and separated by TLC as described in Section 3.8.3. Triacylglycerol synthesis was quantified by measuring the incorporation of radiolabelled [^{3}H]glycerol into triacylglycerol. Radioactivity of the triacylglycerol band was determined as described in Section 3.8.3.

In some experiments, triacylglycerol levels were determined in non-radioactive lipid samples. Lipids were visualized by charring the TLC plate at 180 ºC after immersion in a solution containing 10% cupric sulfate (w/v) and 8% phosphoric acid (v/v). The intensity of triacylglycerol bands were quantified with ImageJ software.

3.10 Cross-linking Studies

Total cellular membranes were extracted from HEK293T cells transiently expressing wild-type DGAT1 and various mutants as described in Section 3.8.1 with the exception that the lysis buffer was 1X PBS containing protease inhibitors. Membrane pellets were resuspended in 200 μL of the lysis buffer, followed by incubation with various concentrations of disuccinimidyl suberate [in DMSO, vehicle concentration 2% (v/v)] at RT for 1 hr. The reactions were terminated with 1/10 volume of 1M Tris-HCl (pH 8.0). Protein samples were then separated by SDS-PAGE and immunoblotted for DGAT1 as described in Section 3.4.3.

3.11 Determination of Protein Stability

To block protein synthesis, HEK293T cells transiently expressing wild-type DGAT1 and various mutants were washed twice with PBS and incubated with fresh culture medium containing 100 μg/mL cycloheximide for 0-12 hr. Cells were harvested at different time points,

followed by extracting total cell lysates as described in Section 3.9. Turnover rate of wild-type DGAT1 and the mutants was determined by Western blot analysis as described in Section 3.4.3.

4.0 RESULTS

4.1 The Role of PKA in Regulating DGAT Activity and Triacylglycerol Synthesis During Lipolysis

Lipolysis is a process by which stored triacylglycerols in white adipose tissue are hydrolyzed to fatty acids and glycerol when blood glucose level decreases. Fatty acids enter the circulation and go to peripheral organs and tissues, where they are oxidized to produce large quantities of ATP. Since lipolysis serves to break down stored triacylglycerols, we investigated what would happen to the triacylglycerol biosynthetic pathway during lipolysis, expecting that triacylglycerol synthesis would be inhibited to promote the efficient net release of stored energy. To test this, we examined DGAT activity during lipolysis in 3T3-L1 adipocytes.

3T3-L1 pre-adipocytes were cultured and differentiated into adipocytes. On day 10 of differentiation, large cytoplasmic lipid droplets were visible. Lipolysis was stimulated by incubating adipocytes with CPT-cAMP, which is a non-hydrolysable cell permeable cAMP analogue that activates PKA. As described in Section 2.9, PKA phosphorylates HSL and perilipin, leading to subsequent activation of HSL and ATGL. It was observed that triacylglycerol levels decreased as the incubation time of adipocytes with CPT-cAMP increased, confirming the activation of lipolytic pathways by CPT-cAMP (Figure 4.1).

To examine the role of PKA in regulating DGAT activity in adipose tissue during lipolysis, *in vitro* DGAT activity in total cellular membranes was measured. Total cellular membranes from adipocytes treated with 0.5 mM CPT-cAMP were pre-incubated with 100 μM of lipase inhibitor, diethyl-p-nitrophenyl phosphate, to minimize degradation of [^{14}C] triacylglycerol formed during the DGAT assay, and assayed for *in vitro* DGAT activity. Unexpectedly, DGAT activity showed an approximately two-fold increase during lipolysis in adipocytes (Figure 4.2), which is opposite to our hypothesis, and also contradicts the previous findings showing that *in vitro* DGAT activity in McArdle RH7777 rat hepatoma cells overexpressing DGAT1 was inhibited by PKA.

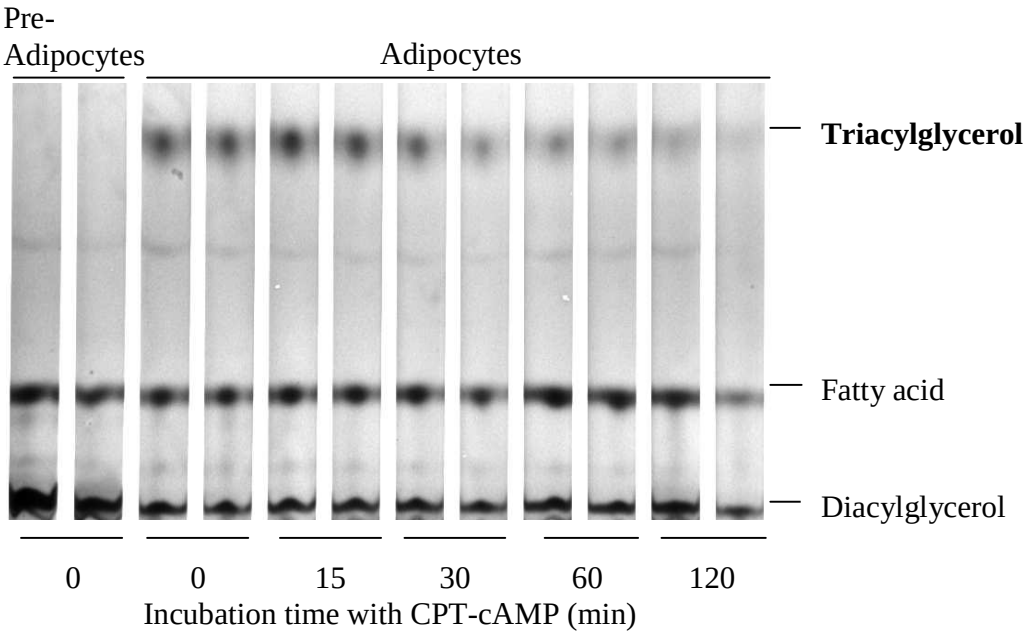


Figure 4.1 Activation of Lipolysis by CPT-cAMP.
3T3-L1 adipocytes were harvested at the indicated incubation times with 0.5 mM CPT-cAMP. Lipids were extracted from total cell lysates and separated by TLC. Lipids were visualized by charring the TLC plate after immersion in cupric sulfate/phosphoric acid. This is a representative result from three independent experiments.

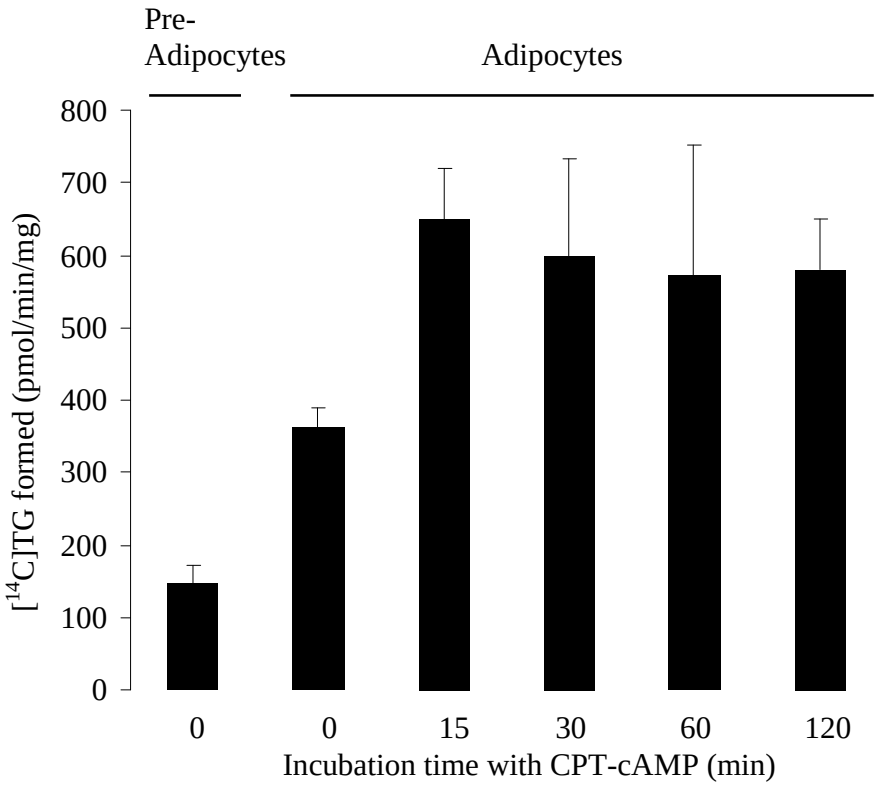


Figure 4.2 Stimulation of DGAT Activity during Lipolysis in Adipocytes.
In vitro DGAT activity assays were performed by measuring activity of 50 μg of protein from total cellular membranes extracted from 3T3-L1 adipocytes incubated with CPT-cAMP for 0-120 min. Pre-adipocytes were used as a control. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. TG: triacylglycerol.

4.2 Mapping the PKA Phosphorylation Site(s) of DGAT1 by Mass Spectrometry

4.2.1 Optimized Immunopurification of DGAT1

As stated in Figure 2.4, DGAT1 has the consensus pattern ([R/K](2)-*X*-[**S/T**]) for PKA at threonine-15 and serine-244. Using site-directed mutagenesis, Stone *et al.* (unpublished observations) previously attempted to map which of these potential sites were phosphorylated by PKA. However, DGAT1 mutants with these residues mutated to alanine residues were still phosphorylated by PKA. Other serine and threonine residues that were considered to be "weak" PKA sites were also mutated. These additional mutants were also still phosphorylated by PKA. Since the results from previous site-directed mutagenesis studies were inconclusive, we decided to use MS to map potential PKA phosphorylation sites in an unbiased manner.

To determine phosphorylation sites within DGAT1 by MS, murine DGAT1 was affinity purified from McArdle RH7777 rat hepatoma cells that stably express FLAG-tagged DGAT1. Although DGAT1 is a hydrophobic membrane protein that has never been purified to homogeneity (Cases *et al*., 1998), Stone *et al.* (unpublished observations) have recently optimized the conditions that allow us to partially purify DGAT1 in an active state from McArdle RH7777 rat hepatoma cells. FLAG-tagged DGAT1 was initially immunoprecipitated from total cell lysate with anti-FLAG agarose beads and eluted with an excess of free FLAG peptide. Prior to incubation with anti-FLAG agarose beads, total cell lysate was solubilized with PBS containing 1% SDS in the presence of phosphatase and protease inhibitors. The immunoprecipitates containing partially purified DGAT1 were separated by SDS-PAGE and immunoblotted for DGAT1. Only a small amount of DGAT1 protein was detected in the starting material after incubation with the anti-FLAG antibody, while the majority of DGAT1 had been successfully immunoprecipitated (Figure 4.3A). Subsequently, to obtain sufficient quantities of DGAT1 for MS analysis, DGAT1 was immunoprecipitated from total cell lysate of 10 100 mm dishes of McArdle RH7777 cells stably expressing FLAG-tagged DGAT1. Following separation by SDS-PAGE, DGAT1 protein could be detected by Coomassie Blue staining (Figure 4.3B). The protein band corresponding to DGAT1 was excised from the gel, and digested with trypsin. The resulting peptides were then identified by MS. Our preliminary MS results suggested that the protein band contained DGAT1, but also over 200 types of contaminating proteins that appeared to be more abundant than DGAT1.

As mentioned in Section 2.5, DGAT1 is present in crude mitochondrial and microsomal fractions. Therefore, to minimize contaminants, FLAG-tagged DGAT1 was immunoprecipitated from membrane factions enriched for DGAT1, which were crude mitochondria and microsomes prepared by subcellular fractionation. To further optimize immunopurification, prior to incubation with anti-FLAG agarose beads, enriched membrane fractions were solubilized with PBS containing two detergents (0.5% SDS and 0.5% Triton X-100) in the presence of phosphatase and protease inhibitors. We managed to eliminate most of the contaminating proteins through the optimized immunopurification and a single band corresponding to DGAT1 was observed (Figure 4.3C).

4.2.2 Identification of PKA Phosphorylation Site(s) of DGAT1 by MS

To determine the PKA phosphorylation sites of DGAT1, affinity purified DGAT1 from Section 4.2.1 was incubated with or without 75 units of PKA. Following the kinase reaction, proteins were separated by SDS-PAGE. The protein band corresponding to DGAT1 was excised from the gel and digested with trypsin. The resulting peptides were subjected to HPLC-ESI-MS/MS analysis to identify potential phosphorylation sites. The molecular weight of each peptide was determined by the first MS analyzer. From the masses of the peptides, a peptide map was generated and compared to a previously constructed data base of murine DGAT1 tryptic fragments, which was incorporated into the data base search engine Mascot (Matrix Science). Peptides matched for DGAT1 yielded an overall protein sequence coverage of 31% (Figure 4.4).

After characterizing DGAT1 using the initial MS analyzer, each peptide entered a collision cell where it was fragmented into individual amino acid residues. The phosphate attached to a peptide was also lost during fragmentation, which could be identified by a neutral loss of 98 daltons, which is the molecular weight of phosphoric acid ($-H_3PO_4$). As a representative example of how specific phosphorylation sites in DGAT1 were assigned, Figure 4.5 shows the tandem mass spectrum of monophosphorylated peptide TG$\mathbf{S}^{17}$RVSVQGGSGPK. This peptide was fragmented in a manner that one amino acid was removed at a time, generating a series of peptide fragments with different molecular weights determined by the second MS analyzer (Figure 4.5A). Peptide fragments with molecular weights of 1197.87 (pink) and 1139.81 (green) daltons showed the neutral loss of 98 daltons respectively, as we

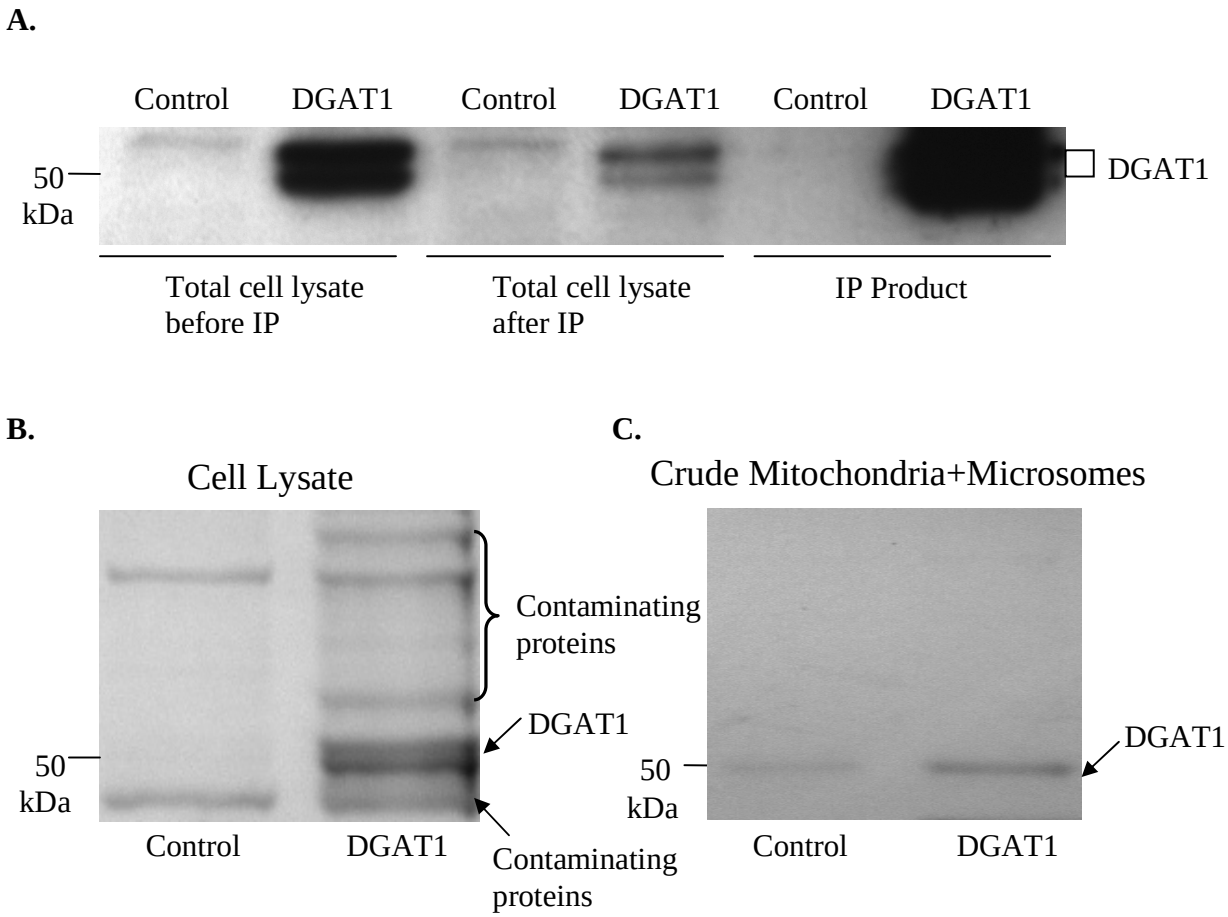


Figure 4.3 Immunopurification of DGAT1.
A: Immunoprecipitation of DGAT1. FLAG-tagged DGAT1 expressed in McArdle RH7777 cells was immunoprecipitated from total cell lysate. Immunoprecipitates were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. McArdle RH7777 cells transfected with the expression vector lacking an insert were used as a control. IP: Immunoprecipitation.
B and C: Coomassie Staining of DGAT1 Before and After Optimizing Its Immunopurification. FLAG-tagged DGAT1 expressed in McArdle RH7777 cells was immunoprecipitated from larger amounts of starting material (cell lysate or crude mitochondria+microsomes). Immunoprecipitates were separated by SDS-PAGE and stained with Coomassie Blue.

compared them with the corresponding fragments generated *in silico* (Figure 4.5B). No more neutral loss was observed in any other pair of peptide fragments, suggesting that serine-17 was the serine that was phosphorylated. Serine-20 and -25 were also identified as potential PKA phosphorylation sites in a similar manner. In agreement with our findings, another independent research group also reported the identification of serine-17 and -20 as phosphorylation sites with >99% certainty (Villén *et al*., 2007).

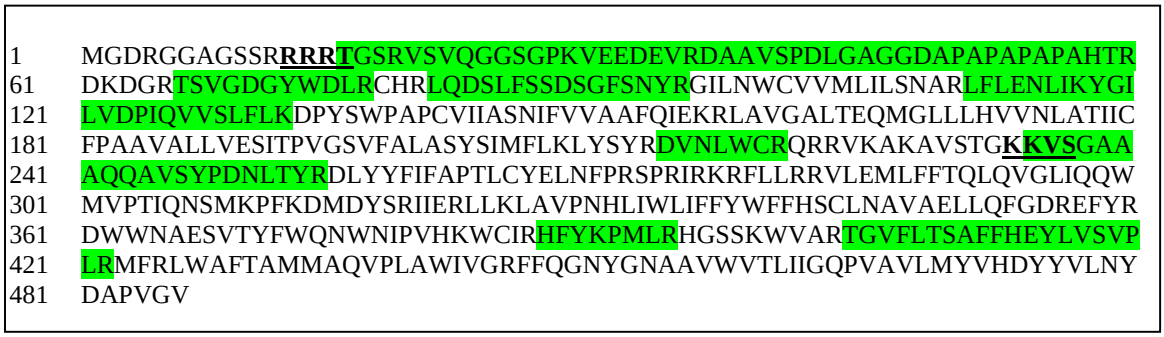
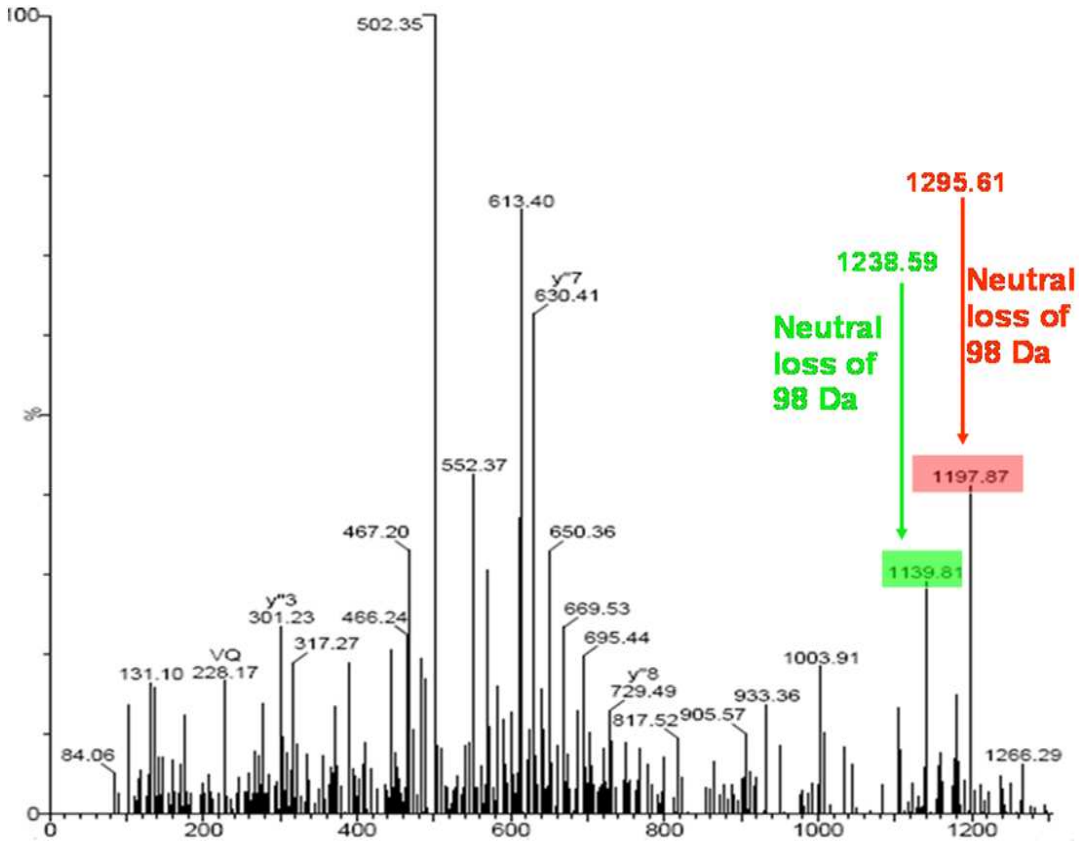


Figure 4.4 Mass Spectrometric Characterization of DGAT1.
Tryptic peptides were subjected to HPLC-ESI-MS/MS analysis. Peptides matched for DGAT1 (highlighted) yielded an overall protein sequence coverage of 31%. DGAT1 has the consensus pattern [R/K](2)-X-[S/T] for PKA phosphorylation site at threonine-15 and serine-244 (underlined and boldfaced).

A.



B:

G	pS	R	V	S	V	Q	G	G	S	G	P	K
13	12	11	10	9	8	7	6	5	4	3	2	1
1295.61	1238.59	1071.59	915.49	816.42	729.39	630.32	502.26	445.24	388.22	301.19	244.17	147.11

Figure 4.5 Mass Spectrometric Analysis of Potential PKA Phosphorylation Sites.
A: MS/MS Spectrum of Peptide TGS[17]RVSVQGGSGPK of DGAT1.
B: *In Silico* Spectrum of Peptide TGS[17]RVSVQGGSGPK of DGATl.
Numbers represent molecular weights of peptide fragments (e.g. 1238.59 is the molecular weight of the fragment SRVSVQGGSGPK).

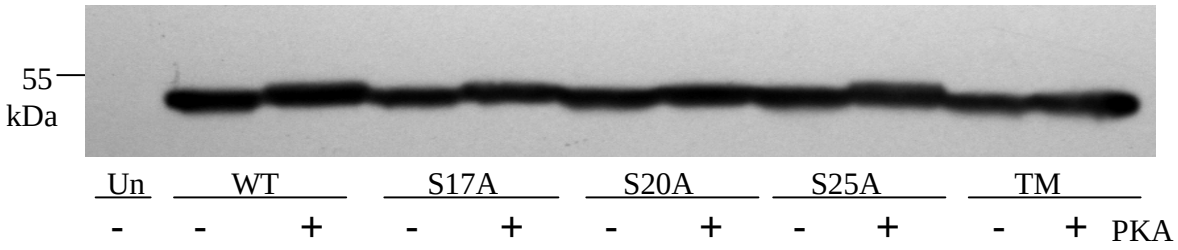
4.3 Determination of Functional Importance of Potential PKA Phosphorylation Sites

4.3.1 The Role of PKA Phosphorylation on DGAT1 Activity and Triacylglycerol Synthesis

To investigate the role of these three potential phosphorylation sites in regulating DGAT1 activity, serine-17, -20, and -25 were mutated to alanine. A triple mutant with all three sites mutated to alanine residues was also generated. DGAT1 mutants, as well as wild-type DGAT1, were transiently expressed in HEK293T cells that are widely used for transient protein expression (Ho and Pastan, 2009). Total cellular membranes were prepared and incubated with or without PKA. Proteins were separated by SDS-PAGE and immunoblotted for DGAT1. Phosphorylation of DGAT1 by PKA led to an increase of the molecular weight of DGAT1, which in turn caused a mobility shift of wild-type DGAT1. Additionally, mobility shifts were observed in the single mutants that are still subject to PKA phosphorylation at the other two unmutated sites. However, the triple mutant displayed little mobility shift (Figure 4.6A), suggesting reduced level of PKA phosphorylation. These results indicate that PKA might phosphorylate DGAT1 at multiple sites. Furthermore, we tested the functional importance of these sites for DGAT activity. *In vitro* DGAT activity assays were performed in the presence of total cellular membranes prepared above. Unexpectedly, *in vitro* DGAT activity of the various DGAT1 mutants appeared to be similar to that of the wild-type DGAT1, which was approximately 12-fold higher than that of untransfected cells, and no inhibition by PKA was observed in either wild-type DGAT1 or the mutants (Figure 4.6B).

We also tested the role of PKA phosphorylation on DGAT1 activity in intact cells. HEK293T cells transiently expressing wild-type DGAT1 or the triple mutant were incubated with the PKA activator, CPT-cAMP, for 0–90 minutes. Total cellular membranes were isolated from transfected cells, proteins were separated by SDS-PAGE and then immunoblotted for DGAT1. Wild-type DGAT1 and the triple-mutant showed similar expression levels and CPT-cAMP treatment did not affect the expression level of DGAT1 (Figure 4.7A). *In vitro* DGAT activity was then measured. Again, wild-type DGAT1 and the triple mutant exhibited similar *in vitro* DGAT activity that was approximately 10 fold higher than that of untransfected cells, and *in vitro* DGAT activity did not change as incubation time with CPT-cAMP increased (Figure 4.7B).

A.



B.

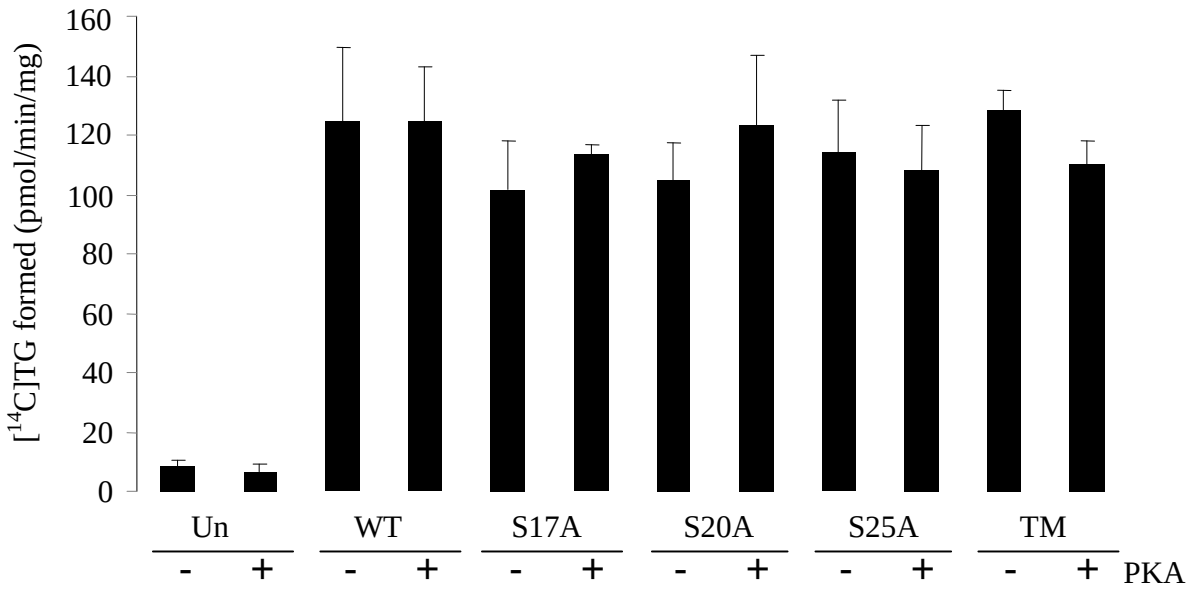


Figure 4.6 The Effect of PKA Treatment on DGAT Activity in HEK293T Cells.
A: Mobility Shift of DGAT1 After PKA Treatment. FLAG-tagged wild-type DGAT1 and DGAT1 mutants were transiently expressed in HEK293T cells, total cellular membranes were incubated with or without 75 units of PKA. Proteins were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. Untransfected HEK293T cells were used as a control. This is a representative result from three independent experiments. Un: untransfected; WT: wild-type; TM: triple mutant.
B: *In vitro* DGAT Activity of Wild-type DGAT1 and DGAT1 Mutants with or without PKA Treatment. *In vitro* DGAT activity assays were performed by measuring activity of 50 μg of protein from total cellular membranes prepared above. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated twice with similar results.
TG: triacylglycerol.

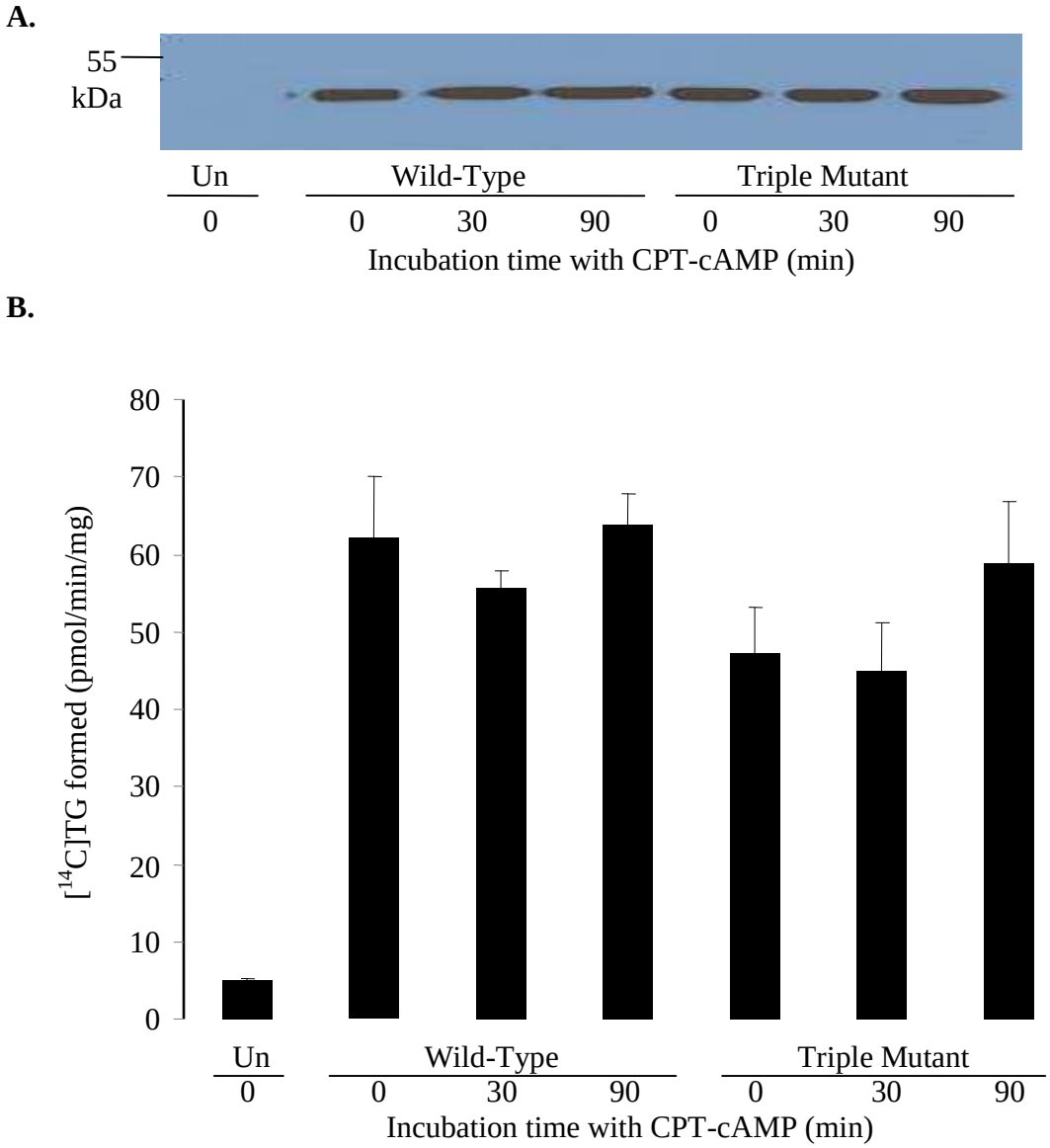


Figure 4.7 The Effect of cAMP Treatment on DGAT Activity.
A: Expression of Wild-type DGAT1 and the Triple Mutant with CPT-cAMP Treatment. HEK293T cells transiently expressing FLAG-tagged wild-type DGAT1 or the triple mutant (S17/20/25A) were incubated with 0.5 mM CPT-cAMP for 0-90 minutes. Total cellular membranes were extracted, proteins were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. Untransfected HEK293T cells were used as control. This is a representative result of three independent experiments. Un: untransfected.
B: *In vitro* DGAT Activity of Wild-type DGAT1 and the Triple Mutant with CPT-cAMP Treatment. *In vitro* DGAT activity assays were performed by measuring activity of 50 μg of protein from total cellular membranes prepared above. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. TG: triacylglycerol.

We further examined the functional importance of these putative phosphorylation sites for triacylglycerol synthesis in intact cells. HEK293T cells transiently expressing wild-type DGAT1 or the triple mutant were metabolically labeled with 10 μCi [^{3}H]glycerol for 6 hours to determine the amount of newly synthesized triacylglycerol. Lipids were extracted from cell lysates and separated by TLC. Although wild-type DGAT1 and the triple mutant showed an approximately 2-fold increase in triacylglycerol synthesis compared to untransfected cells, similar triacylglycerol synthesis was observed from wild-type DGAT1 and the triple mutant (Figure 4.8).

Since the inhibitory effect of PKA on DGAT1 activity and triacylglycerol synthesis was initially observed in McArdle RH7777 liver cells stably expressing DGAT1 (Stone *et al*., unpublished observations), we suspected that the role PKA had on DGAT1 activity and triacylglycerol synthesis might be cell type dependent. Therefore, instead of investigating functional importance of these PKA sites for DGAT1 activity and triacylglycerol synthesis in HEK293T cells, wild-type DGAT1 and the triple mutant were transiently expressed in McArdle RH7777 liver cells. Total cellular membranes were prepared, and western blot analysis was performed to determine protein expression levels of wild-type DGAT1 and the triple mutant. It was observed that they both were equally expressed in McArdle RH7777 cells (Figure 4.9A). *In vitro* DGAT activity assays were carried out in the presence of total cellular membranes pre-incubated with or without PKA. Although *in vitro* DGAT activity of wild-type DGAT1 and the triple mutant was approximately 3-fold higher than that of untransfected cells, *in vitro* DGAT activity showed no difference between wild-type DGAT1 and the triple mutant in the presence or absence of PKA (Figure 4.9B).

Since previous studies have demonstrated that DGAT1 exists as a homotetramer (Cheng *et al*., 2001), endogenous DGAT1 in mammalian cells could potentially oligomerize with recombinant DGAT1 and confound our studies. Therefore, DGAT activity of the triple mutant was also studied in a mutant yeast strain (H1246MATα) that lacked all four genes contributing to triacylglycerol synthesis in yeast, including the gene encoding DGAT1 (Sandager *et al*., 2001). The cDNA encoding FLAG-tagged DGAT1 was cloned into a yeast expression vector pYES2 containing a galactose inducible *GAL1* promoter. A triple mutant construct with all three putative phosphorylation sites mutated to alanine residues was also generated. The yeast strain H1246MATα was transformed with either construct. Expression of DGAT1 was induced

with galactose for 24 hr. Transformants were then lysed and total cellular membranes were extracted. Western blot analysis was carried out to examine protein expression levels. Wild-type DGAT1 and the triple mutant were expressed at similar levels (Figure 4.10A). *In vitro* DGAT activity assays were performed using cellular membranes pre-incubated with or without PKA. It was observed that both wild-type DGAT1 and the triple mutant were similarly active when expressed in yeast (Figure 4.10B), and showed increased DGAT activity compared to untransfected cells (~7-fold). In addition, there was no apparent difference in DGAT activity between PKA treated and non-PKA treated samples.

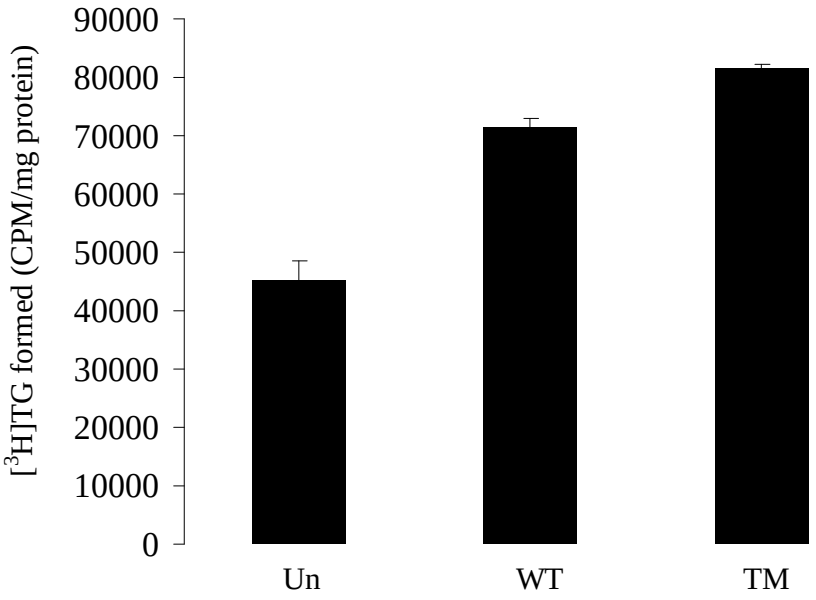


Figure 4.8 *De novo* Triacylglycerol Synthesis from Wild-type DGAT1 and the Triple Mutant in HEK293T Cells.
HEK293T cells transiently expressing wild-type DGAT1 or the triple mutant (S17/20/25A), as well as untransfected HEK293T cells, were incubated with 10 µCi [^{3}H]glycerol and 0.375mM oleic acid complexed to 0.5% BSA for 6 hours. Lipids were extracted from cell lysates and separated by TLC. The incorporation of radioactivity into triacylglycerol was quantified by liquid scintillation counting and normalized to total cellular membrane ***protein content***. Data are mean $\pm$ S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. Un: untransfected; WT: wild-type; TM: triple mutant; TG: triacylglycerol.

A.

B.

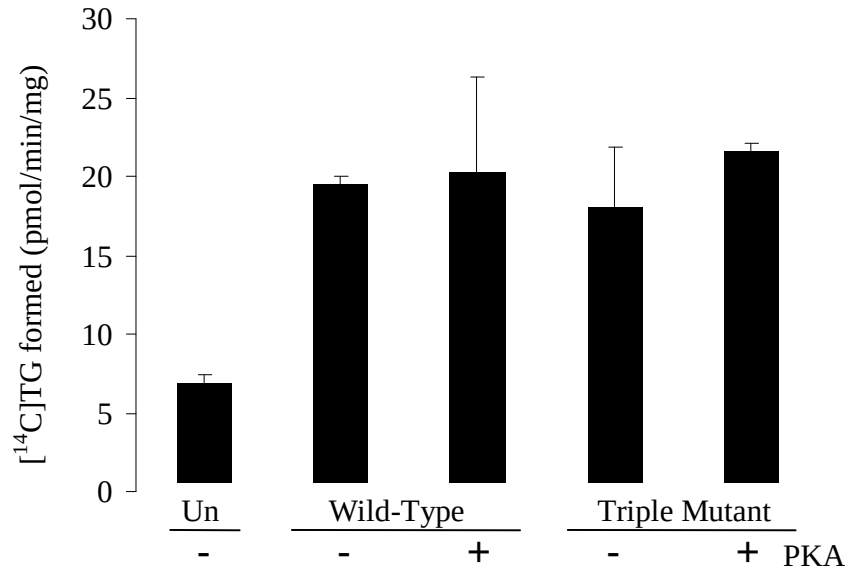


Figure 4.9 The Effect of PKA Treatment on DGAT Activity in McArdle RH7777 Hepatoma Cells.

A: Expression of Wild-type DGAT1 and the Triple Mutant in McArdle RH7777 Liver Cells. Total cellular membranes were extracted from McArdle cells transiently expressing FLAG-tagged wild-type DGAT1 or the triple mutant (S17/20/25A). Proteins were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. Untransfected McArdle cells were used as control. This is a representative result from three independent experiments. Un: untransfected. * Non-specific protein band.

B: *In vitro* DGAT Activity of Wild-type DGAT1 and the Triple-mutant with or without PKA Treatment. *In vitro* DGAT activity assays were performed by measuring activity of 100 μg of protein from total cellular membranes pre-incubated with or without 75 units of PKA. Data are mean $\pm$ S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. TG: triacylglycerol.

A.

B.

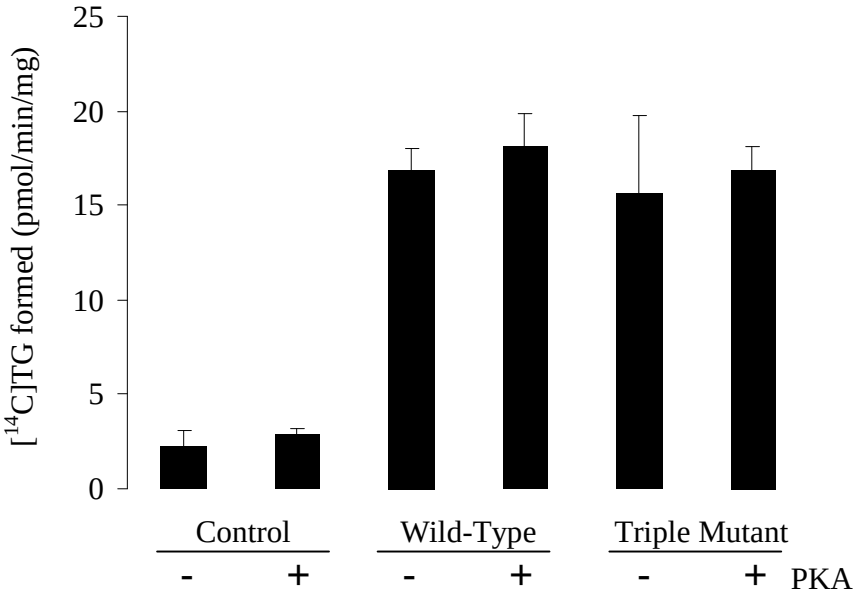


Figure 4.10 The Effect of PKA Treatment on DGAT Activity in the Yeast Strain H1246MATα.

A: Expression of Wild-type DGAT1 and the Triple Mutant in the Yeast Strain H1246MATα. Total cellular membranes were extracted from yeast cells expressing FLAG-tagged wild-type DGAT1 or the triple mutant (S17/20/25A). Proteins were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. Yeast cells harbouring an empty pYES2 vector were used as a control. This is a representative result of three independent experiments.

B: *In vitro* DGAT Activity of Wild-type DGAT1 and the Triple Mutant with or without PKA Treatment. *In vitro* DGAT activity assays were performed by measuring activity of 200 µg of protein from total cellular membranes pre-incubated with or without 75 units of PKA. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. TG: triacylglycerol.

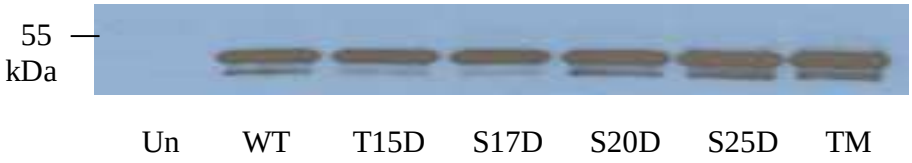
4.3.2 The Role of Phosphorylation on DGAT1 Activity

Since our mutagenesis approach where candidate phosphorylation sites were mutated to alanines was not informative, we chose to generate DGAT1 variants that were "permanently" phosphorylated. Serine-17, -20, and -25 were mutated to aspartate, whose negative charge mimics the phosphorylated state of a protein. A triple mutant with all three sites mutated to aspartates was also generated. In addition, threonine 15, which had been considered a strong candidate for phosphorylation site and rendered DGAT1 hyperactive when mutated to alanine (Stone *et al*., unpublished observations), was mutated to aspartate as well. DGAT1 mutants as well as wild-type DGAT1 were transiently expressed in HEK293T cells, total cellular membranes were prepared and proteins were separated by SDS-PAGE and immunoblotted for DGAT1. Wild-type DGAT1 and mutants were expressed at similar levels (Figure 4.11A). *In vitro* DGAT activity assays were performed. Again, no significant difference in DGAT activity was observed between wild-type and the aspartate mutants (Figure 4.11B).

4.3.3 The Role of PKA Phosphorylation on Multimer Formation of DGAT1

All the results suggested that these putative phosphorylation sites were not critical for DGAT1 activity and triacylglycerol synthesis, indicating that they may be involved in regulating other aspects of DGAT1. Previous studies have illustrated that the N-terminal region of DGAT1 is responsible for DGAT1 to form homodimers and homotetramers (Cheng *et al*., 2001). Dephosphorylation of DGAT1 with phosphatase converted homotetramers/dimers of DGAT1 to monomers (Stone *et al*., unpublished observations). Therefore, we decided to test whether these phosphorylation sites residing at the N-terminus played a role in regulating DGAT1 multimer formation. Wild-type DGAT1, the triple alanine mutant, and the triple aspartate mutant were transiently expressed in HEK293T cells. Total cellular membranes were prepared and incubated with various concentrations of DSS, which is a homobifunctional, membrane permeable, and amine-reactive cross-linker, for 20 min. Protein samples were then separated by SDS-PAGE and immunoblotted for DGAT1. Both the triple alanine mutant and the triple aspartate mutant were still capable of forming dimers and tetramers as wild-type DGAT1 was (Figure 4.12), indicating that these phosphorylation sites are not required for regulating interactions of DGAT1 subunits.

A.



B.

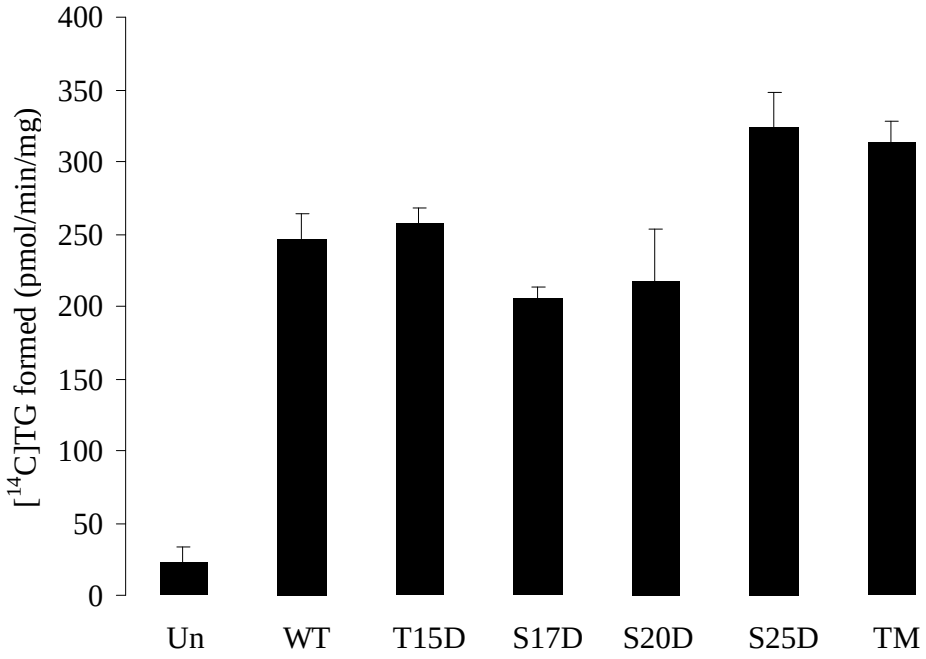


Figure 4.11 DGAT Activity of Wild-type DGAT1 and Aspartate Mutants in HEK293T Cells.

A: Expression of Wild-type DGAT1 and Aspartate Mutants. Total cellular membranes were extracted from HEK293T cells transiently expressing FLAG-tagged wild-type DGAT1 or aspartate mutants. Proteins were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. Untransfected HEK293T cells were used as a control. This is a representative result of three independent experiments. Un: untransfected; WT: wild-type; TM: triple mutant.

B: *In vitro* DGAT Activity of Wild-type DGAT1 and Aspartate Mutants. *In vitro* DGAT activity assays were performed by measuring activity of 50 μg of protein from total cellular membranes prepared above. Data are mean $\pm$ S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. TG: triacylglycerol.

4.3.4 The Role of PKA Phosphorylation on DGAT1 Stability

Furthermore, we examined whether these PKA phosphorylation sites played a role in regulating protein stability of DGAT1. Wild-type DGAT1 constructs, as well as the triple alanine mutant and the triple aspartate mutant, were transiently expressed in HEK293T cells. 24 hr post-transfection, cells were treated with 100 µg/ml cycloheximide to block protein synthesis (Yu *et al*., 2002a). To determine the turnover rate of wild-type DGAT1 and the mutants, cells were analyzed for the protein level of DGAT1 by Western blot analysis at various time points after cycloheximide treatment. 12 hr post cycloheximide incubation, wild-type DGAT1, as well as both triple mutants, did not display any alteration of protein level (Figure 4.13), indicating that DGAT1 is very stable (Yu *et al*., 2002a) and these phosphorylation sites do not have a significant role in the control of DGAT1 turnover.

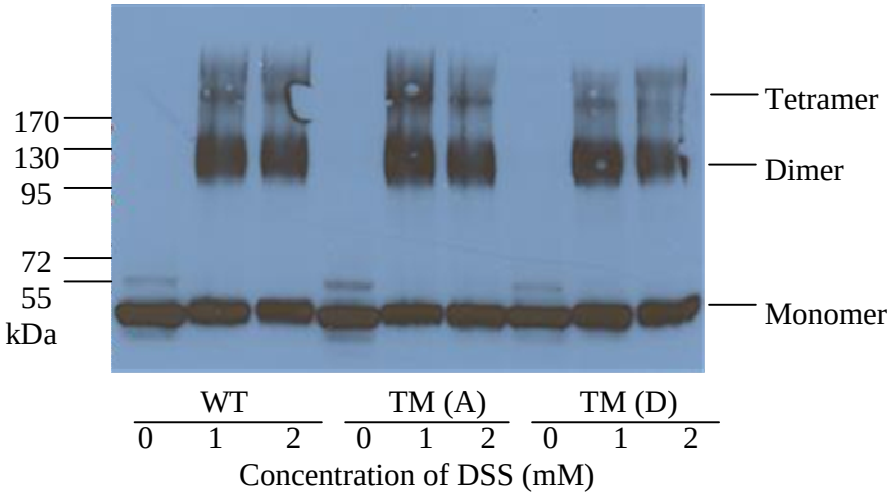


Figure 4.12 Cross-linking of Wild-type DGAT1 and Triple Mutants with DSS.
Flag-tagged wild-type DGAT1, the triple alanine mutant (S17/20/25A), and the triple aspartate mutant (S17/20/25D) were transiently expressed in HEK293T cells. Total cellular membranes were prepared and incubated with various concentrations of DSS [in DMSO, vehicle concentration 2% (v/v)] for 20 min at room temperature. The reactions were quenched with 1M Tris, pH 8.0 (1/10 volume of reaction mixture). Reaction mixtures were separated by SDS-PAGE and immunoblotted for DGAT1 using a monoclonal anti-FLAG antibody. This is a representative result of two independent experiments. WT: wild-type; TM (A): triple alanine mutant; TM (D): triple aspartate mutant.

4.3.5 The Role of PKA Phosphorylation on Substrate Affinity of DGAT1

Previous studies have demonstrated that deleting the first 65 amino acids at the N-terminus of ACAT1, which is homologous to DGAT1, resulted in decreased affinity for oleoyl-CoA (Yu *et al*., 2002b). Although our data suggested that these phosphorylation sites were not involved in regulating Vmax of DGAT1, they might have a role in affecting substrate affinity of DGAT1. Therefore, wild-type DGAT1, as well as the triple alanine mutant, were transiently expressed in HEK293T cells. Total cellular membranes were prepared. *In vitro* DGAT activity assays were carried out with various concentrations of dioleoylglycerol substrate to establish a dioleoylglycerol substrate affinity curve of DGAT1. Wild-type DGAT1 and the triple alanine mutant exhibited similar Vmax in the presence of both endogenous and exogenous diacylglycerols (Figure 4.14), which is consistent with our previous findings. However, wild-type DGAT1 showed higher activity in the presence of endogenous diacylglycerols alone, suggesting that wild-type DGAT1 may have higher affinity towards particular endogenous diacylglycerol(s). Comparing to the triple alanine mutant, wild-type DGAT1 is still subject to PKA phosphorylation, which indicates that PKA phosphorylation may have a role in increasing the substrate affinity of DGAT1.

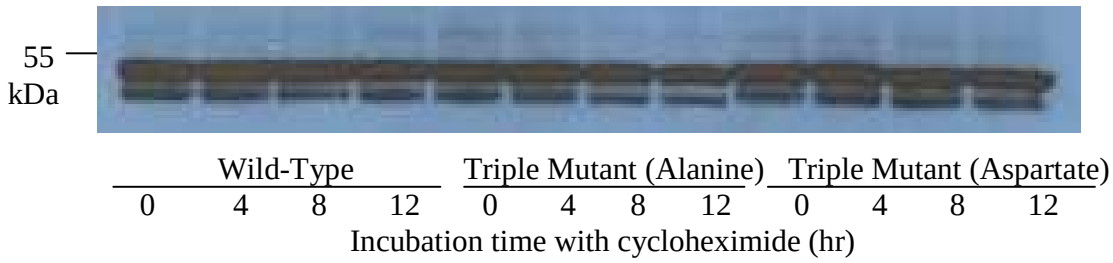


Figure 4.13 Turnover Rate of Wild-type DGAT1 and Triple Mutants.
FLAG-tagged wild-type DGAT1, as well as the triple alanine mutant (S17/20/25A) and the triple aspartate mutant (S17/20/25D), were transiently expressed in HEK293T cells. 24 hr post-transfection, cells were subjected to cycloheximide treatment for various periods of time followed by assessment of protein level. This is a representative result of two independent experiments.

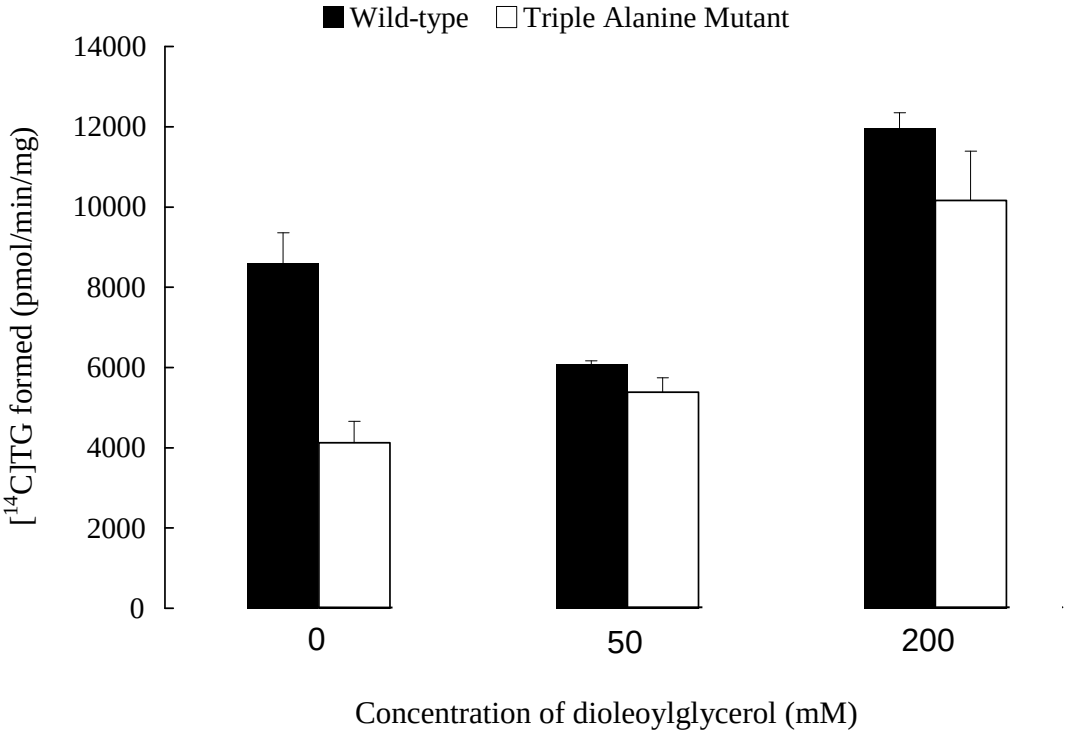


Figure 4.14 Substrate Affinity of Wild-type DGAT1 and the Triple Alanine Mutant.
Total cellular membranes were extracted from HEK293T cells transiently expressing wild-type DGAT1 or the triple alanine mutant (S17/20/25A). *In vitro* DGAT activity assays were performed by measuring activity of 50 μg of protein from total cellular membranes with various concentrations of dioleoylglycerol. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated once with similar results. TG: triacylglycerol.

5.0 DISCUSSION

5.1 DGAT1 Recycling Hypothesis

As stated in Section 2.9, the first objective of this thesis was to determine the role of PKA in regulating DGAT1 activity and triacylglycerol synthesis during lipolysis. It was hypothesized that triacylglycerol synthesis and breakdown are coordinately regulated processes. Therefore, during lipolysis, a period of triacylglycerol breakdown, the rate of triacylglycerol synthesis in adipose tissue was expected to decrease in order to promote the efficient net release of stored energy. Unexpectedly, *in vitro* DGAT activity showed a two-fold increase during lipolysis in adipocytes, which is opposite to our hypothesis. This also contradicts previous findings that showed *in vitro* DGAT1 activity of liver or McArdle RH7777 rat hepatoma cells overexpressing DGAT1 could be inhibited by PKA.

However, the increased DGAT activity during lipolysis was consistent with the fatty acid recycling hypothesis. Studies in both animals and humans demonstrated that only 30% of the free fatty acids released from triacylglycerol lipolysis in white adipose tissue are delivered to peripheral tissues, such as liver and muscle (Reshef *et al*., 2003). The other 70% of free fatty acids re-enter adipocytes and are re-esterified to triacylglycerols (Figure 5.1). Further studies demonstrated that such recycling occurs not only in adipose tissue but also in liver and skeletal muscle (Prentki *et al*., 2008). In addition, it is important to note that the percentage of lipolysis-released free fatty acids that is recycled back to triacylglycerols is relatively constant (~70%) under different metabolic conditions (Reshef *et al*., 2003; Prentki *et al*., 2008). In response to the increased supply of fatty acids during lipolysis, DGAT1 activity could be stimulated to increase triacylglycerol synthesis to prevent free fatty acids from reaching toxic levels and producing membrane-damaging effects within the cell. Moreover, these results are consistent with the previous findings showing that DGAT2 is more responsible for promoting bulk synthesis of triacylglycerols, while DGAT1 plays more of a role in regulating energy metabolism (Figure 5.1) (Stone *et al*., 2004).

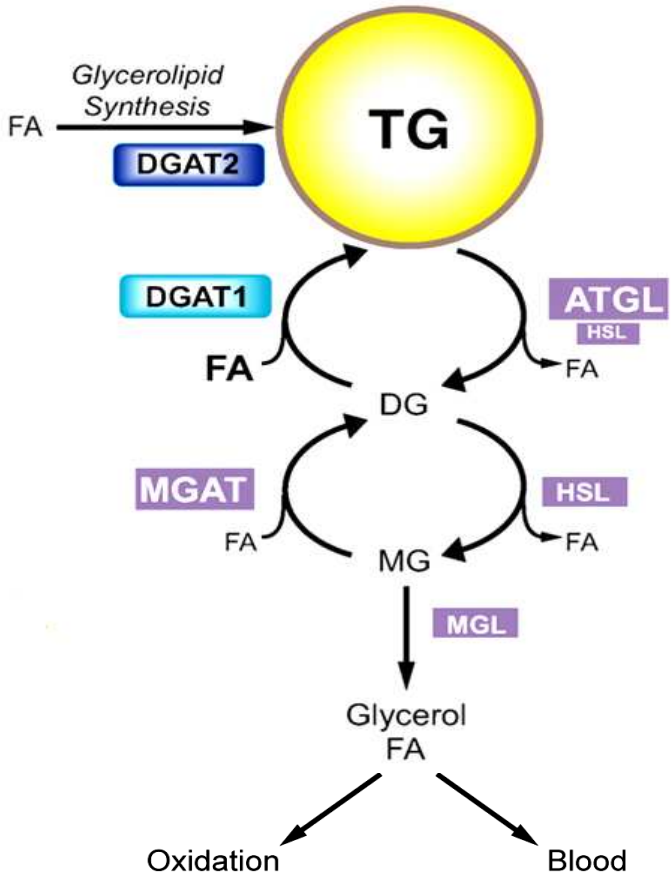


Figure 5.1 DGAT1 Recycling Hypothesis.
Based on the available evidence, DGAT1 is speculated to be involved in the recycling of hydrolyzed triacylglycerols by re-esterifying fatty acids, which in turn prevents fatty acids from reaching toxic levels and producing membrane-damaging effects within the cell. On the other hand, DGAT2 is more responsible for promoting bulk synthesis of triacylglycerols. HSL, hormone-sensitive lipase; ATGL, adipose-tissue triglyceride lipase; MGL, monoacylglycerol lipase; TG: triacylglycerol; DG: diacylglycerol; FA: fatty acid. The diagram is modified from Yen *et al.* (2008).

5.2 Existence of Other Possible PKA Phosphorylation Sites

Since preliminary studies have identified PKA as one possible kinase that could inhibit DGAT1 activity, the major focus of this thesis was to map PKA phosphorylation sites of DGAT1 and determine their functional importance with respect to triacylglycerol synthesis. The approach was to identify PKA phosphorylation sites by analyzing trypsin digests of affinity purified DGAT1 by HPLC-ESI-MS/MS. Peptides matched for DGAT1 yielded a protein sequence coverage of 31%. Serine-17, -20 and -25 of DGAT1 were identified as potential PKA phosphorylation sites. Although these three sites do not fit the PKA consensus sequence R/K-R/K-X-S/T (Taylor et al., 1990), serine-17 was previously considered as a "weak" PKA site. It has been demonstrated that the presence of a consensus phosphorylation site does not guarantee that the protein is a substrate *in vivo*, and authentic phosphorylation sites do not always conform to the consensus (Ubersax *et al*., 2003). Villén *et al* (2007) also carried out large-scale phosphorylation analysis of mouse liver through a combination of tandem phosphopeptide enrichment methods, high performance MS, and optimized database search/data filtering strategies. In agreement with our findings, they also reported that serine-17 and -20 of DGAT1 were phosphorylated with >99% certainty.

To determine if serine-17, -20 or -25 had a role in regulating DGAT1 activity and triacylglycerol synthesis, we generated DGAT1 mutants in which these amino acids were replaced with alanine, and expressed them in mammalian and yeast expression systems respectively.

In HEK293T cells transiently expressing wild-type DGAT1 or various alanine mutants, wild-type DGAT1 and single mutants displayed mobility shift caused by PKA phosphorylation. However, little shift was observed in the triple mutant with all three PKA sites mutated to alanines, suggesting reduced level of PKA phosphorylation. These results confirm the locations of putative PKA phosphorylation sites identified by MS. These data also indicates that DGAT1 likely has multiple phosphorylation sites, which is supported by the mass spectrometric identification of one tryptic peptide with both serine-20 and -25 being phosphorylated. From our preliminary data, DGAT1 mutants lacking functional PKA sites were expected to be resistant to inhibition by PKA, and would have higher DGAT activity and triacylglycerol synthesis compared to wild-type DGAT1. However, *in vitro* DGAT activity of the various DGAT1 mutants appeared to be similar to that of the wild-type DGAT1, and no inhibition by

PKA was observed in either wild-type DGAT1 or the mutants. In HEK293T cells transiently expressing wild-type DGAT1 or the triple mutant, DGAT activity of intact cells could not be inhibited by treating cells with the PKA activator, CPT-cAMP. Furthermore, *de novo* triacylglycerol synthesis was measured in intact cells by metabolically labeling cells with [^{3}H]glycerol, and wild-type DGAT1 and the triple mutant exhibited similar level of triacylglycerol synthesis. In McArdle RH7777 rat hepatoma cells, where the inhibitory effect of PKA on DGAT activity and triacylglycerol synthesis had been initially observed (Stone *et al.*, unpublished data), *in vitro* DGAT1 activity was not different between wild-type DGAT1 and the triple mutant in the presence or absence of PKA. All these results suggested that PKA phosphorylation at serine-17, -20 and -25 has no apparent effect on the ability of DGAT1 to synthesize triacylglycerols.

All mammalian cells express DGAT1 which may be confounding our studies. Therefore, DGAT activity of the triple mutant was also studied in a mutant yeast strain (H1246MATα) that lacked all the enzymes that are capable of catalyzing triacylglycerol formation. Wild-type DGAT1 and the triple mutant still displayed similar DGAT1 activity, and no PKA inhibition was observed. These results were consistent with our findings in HEK293T cells.

In addition to analyzing mutations mimicking the dephosphorylated state of DGAT1, mutations of threonine-15, serine-17, -20, and -25 to aspartate that mimic the phosphorylated state were also examined in HEK293T cells. As mentioned in Section 4.3.2, threonine-15 was included because it has the consensus sequence for a PKA phosphorylation site and rendered DGAT1 hyperactive when mutated to alanine. According to our hypothesis and all the preliminary data, DGAT1 mutants mimicking the phosphorylated state of the protein were expected to be inhibited, and would have reduced DGAT activity compared to wild-type DGAT1. However, *in vitro* DGAT activity of the various DGAT1 mutants appeared to be similar to that of wild-type DGAT1, which is consistent with the data suggesting that phosphorylation at serine-17, -20 and -25 has no apparent effect on DGAT1 activity.

Although all the results differ from our expectations, they are consistent with those from a recent study by McFie *et al.* (2010), where a particular N-terminal region between amino acids 38-84, but not 2-37 of DGAT1, appeared to play a role in regulating DGAT1 activity. McFie *et al.* showed that DGAT activity of a DGAT1 mutant lacking amino acids 2-37 was

comparable to that of wild-type DGAT1, whereas DGAT activity of the mutant lacking amino acids 2-84 was ~14 fold higher than that of wild-type DGAT1. We speculate that serine-17, -20, and -25 are functionally silent in terms of regulating DGAT1 activity and triacylglycerol, and PKA might inhibit DGAT1 activity via other unmapped, functionally active phosphorylation site(s) between amino acids 38-84. There are a few serine/threonine amino acids in this region. Serine-67 is a potential PKA phosphorylation site as DGAT1 has a weak consensus sequence at this amino acid (G-R-T-**S**^{67}).

We further investigated whether the three putative phosphorylation sites were involved in regulating other aspects of DGAT1 other than activity. Through cross-linking studies, we found that these phosphorylation sites were not required for regulating interactions of DGAT1 subunits. Both the triple alanine mutant and the triple aspartate mutant were still capable of forming dimers and tetramers as wild-type DGAT1 was. These findings are consistent with the data showing that amino acids 37-84, not 2-37 of DGAT1, are required for DGAT1 tetramer formation (McFie *et al*., 2010). McFie *et al.* also speculated that DGAT1 activity may be regulated by switching between an active dimer and an inactive tetramer as the inability of DGAT1 to form a tetramer corresponded to a several-fold increase in *in vitro* enzyme activity. Taken together, these results indicate that PKA may regulate DGAT1 activity by converting it from an active dimer to an inactive tetramer via protein phosphorylation at the region between amino acids 37-84.

Through inhibition of *de novo* protein synthesis, we also examined whether serine-17, -20 or -25 played a role in regulating DGAT1 stability. It was observed that 12 hours after inhibiting protein synthesis with cycloheximide, wild-type DGAT1, as well as both triple mutants, did not exhibit any alteration of protein level as determined by immunoblotting. These data are consistent with the previous findings showing that DGAT1 is very stable (Yu *et al*., 2002), and also suggest that these three sites are not significant factors in the control of DGAT1 turnover.

Lastly, although phosphorylation at the three putative sites has no effect on DGAT1 activity measured under apparent Vmax conditions described in Section 3.8.3, it played a role in regulating the substrate affinity of DGAT1. Wild-type DGAT1 and the triple alanine mutant exhibited similar Vmax in the presence of both endogenous and exogenous diacylglycerols, which is in accordance with our previous findings. However, wild-type DGAT1 showed higher

activity with endogenous diacylglycerols alone, indicating that wild-type DGAT1 had higher affinity towards endogenous diacylglycerols. Comparing to the triple alanine mutant, wild-type DGAT1 is still subject to PKA phosphorylation. Therefore, PKA phosphorylation plays a role in increasing the substrate affinity of DGAT1.

5.3 Presence of Other Protein Kinases that Phosphorylate DGAT1

As mentioned in Section 2.7, preliminary experiments demonstrated that DGAT1 could be inactivated by a kinase activity present in the cytosol. Although PKA was proposed to be a strong candidate, we cannot exclude the possibility that DGAT1 is phosphorylated and regulated by other protein kinases. As depicted in Figure 4, DGAT1 has several potential PKC phosphorylation sites, one AMPK site and one tyrosine kinase site. In fact, studies performed with protein kinase inhibitors suggested that the inhibition of DGAT activity was due to tyrosine kinase activity (Lau *et al*., 1996). Although mutagenesis experiments indicated that this potential tyrosine phosphorylation site did not appear to have a role in regulating DGAT activity (Yu *et al*., 2002), there might be other tyrosine residues that are phosphorylated and potentially regulate DGAT activity. Moreover, Assifi *et al.* (2005) reported a two-fold increase of DGAT activity in rat liver associated with a decrease in AMPK activity during refeeding after a fast, prompting speculation that AMPK might be involved in regulating DGAT1 activity through phosphorylation. In addition, the inability of PKA to exert effect on DGAT1 activity through the identified phosphorylation sites indicates that DGAT1 is the target of other protein kinases.

5.4 Conclusions

Recent studies have shed light on the metabolic functions of DGAT enzymes, however, little is known about the mechanisms responsible for regulating DGAT activity. Several lines of evidence from previous studies indicate that DGAT1, but not DGAT2, is subject to regulation by phosphorylation and that protein kinase A (PKA)-dependent pathways are likely involved. In this thesis, we hypothesized that during lipolysis in adipose tissue, PKA would phosphorylate and inactivate DGAT1, which in turn would facilitate the efficient net release of stored energy by decreasing triacylglycerol synthesis. In contrast, our experimental results showed that *in vitro* DGAT activity increased 2 fold during lipolysis in 3T3-L1 adipocytes.

This data suggests that DGAT activity might increase during lipolysis to promote the recycling/re-esterification of excessive free fatty acids into triacylglycerols before they reach toxic levels within the cell.

To further explore the role of PKA in regulating DGAT1, PKA phosphorylation sites of DGAT1 were mapped by HPLC-ESI-MS/MS. Serine-17, -20 and -25 were identified as potential PKA phosphorylation sites. Moreover, the functional importance of these three potential phosphorylation sites was investigated. Mutations of these sites to alanine or aspartate residues gave rise to enzymes functioning similarly to wild-type DGAT1. These phosphorylation sites did not appear to be involved in regulating DGAT1 activity, multimer formation, or protein stability. However, PKA phosphorylation at these three sites seemed to play a role in affinity of DGAT1 for its diacylglycerol substrate. These results suggest the existence of other unidentified, functionally active PKA phosphorylation sites or phosphorylation sites of other kinases, which are involved in regulating DGAT1 activity and triacylglycerol synthesis.

5.5 Future Directions

Unidentified PKA phosphorylation sites should be continuously mapped and examined in the same manner as described in this thesis. Furthermore, to identify potential phosphorylation sites of other kinases, DGAT1 should be incubated with mouse liver cytosol, in the presence of ATP and magnesium, to promote protein phosphorylation. We should then map the regions of DGAT1 that are phosphorylated in an unbiased manner by HPLC-ESI-MS/MS. We should examine the phosphorylation sites and compile a list of candidate kinases to test, based on known protein kinase consensus sequences. The roles of candidate kinases in regulating DGAT1 should then be assessed by site-directed mutagenesis experiments.

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