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Review

Enzymes of triacylglycerol synthesis and their regulation

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Abstract

Since the pathways of glycerolipid biosynthesis were elucidated in the 1950's, considerable knowledge has been gained about the enzymes that catalyze the lipid biosynthetic reactions and the factors that regulate triacylglycerol biosynthesis. In the last few decades, in part due to advances in technology and the wide availability of nucleotide and amino acid sequences, we have made enormous strides in our understanding of these enzymes at the molecular level. In many cases, sequence information obtained from lipid biosynthetic enzymes of prokaryotes and yeast has provided the means to search the genomic and expressed sequence tag databases for mammalian homologs and most of the genes have now been identified. Surprisingly, multiple isoforms appear to catalyze the same chemical reactions, suggesting that each isoform may play a distinct functional role in the pathway of triacylglycerol and phospholipid biosynthesis. This review focuses on the *de novo* biosynthesis of triacylglycerol in eukaryotic cells, the isoenzymes that are involved, their subcellular locations, how they are regulated, and their putative individual roles in glycerolipid biosynthesis.

Contents

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1. Introduction

Bacteria, yeast, plants, and animals all have the ability to synthesize triacylglycerol (TAG), a critical function during both periods of nutritional excess and nutritional stress. In eukaryotes TAG is an energy store and a repository of essential and non-essential fatty acids and precursors for phospholipid biosynthesis. Fatty acids are packaged in VLDL and chylomicra as TAG for distribution to peripheral tissues where they can be used immediately or stored. TAG is also an essential component of milk (Fig. 1, pathway 1). In addition, forming TAG acts to attenuate

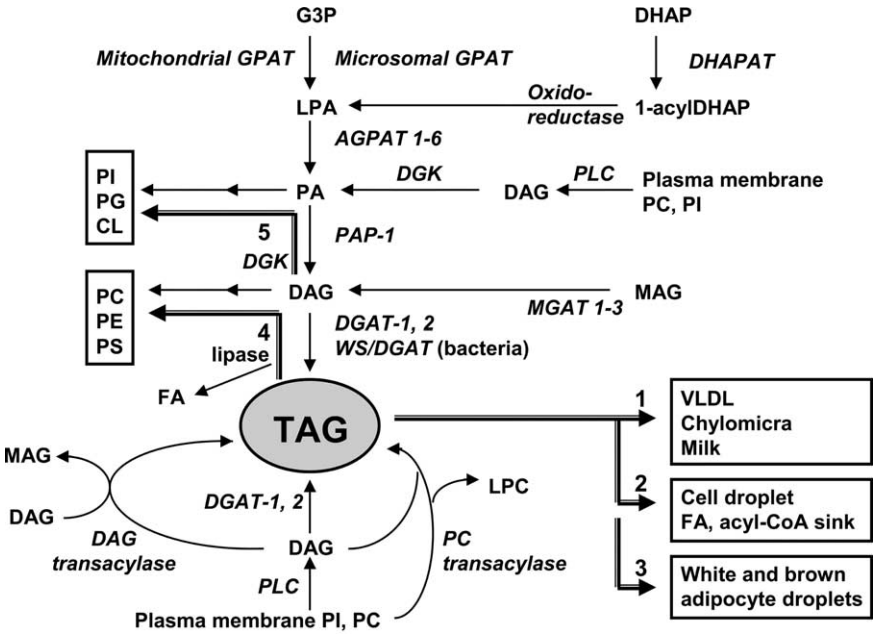

Fig. 1. Synthesis of triacylglycerol and its metabolic pathways. Pathway 1, synthesis of VLDL, chylomicra, milk; Pathway 2, removal of potentially toxic fatty acids and acyl-CoAs; Pathway 3, energy storage in adipocyte droplets; Pathway 4, Provision of diacylglycerol for phospholipid synthesis; and Pathway 5, provision of phosphatidate for phospholipid synthesis. AGPAT, acylglycerol-P acyltransferase; CL, cardiolipin; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGK, diacylglycerol kinase; DHAP, dihydroxyacetone-P; DHAPAT, DHAP acyltransferase; FA, fatty acid; G3P, glycerol-3-phosphate; GPAT, glycerol-P acyltransferase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLC, phospholipase C; PS, phosphatidylserine; TAG, triacylglycerol; WS, wax ester synthase.

diacylglycerol (DAG) signals and to protect cells from sudden increases in fluxes of fatty acids and acyl-CoAs (Fig. 1, Pathway 2). It is generally believed that these molecules are potentially membrane-damaging and that their incorporation into TAG enables them to be converted to a non-toxic compound that can be safely stored. Thus, even brain tissue has the ability to synthesize TAG [1]. TAG is contained in lipid droplets in every cell in which it has been looked for. Like the huge droplets in fat cells, these smaller droplets are enclosed by a monolayer of phospholipid and specific proteins like ADRP that probably regulate their formation, growth, and dissolution (Fig. 1, pathway 3) [2,3]. Finally, TAG stores can be partially hydrolyzed to form DAG, a precursor of the major phospholipids phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (Fig. 1, pathway 4), and the DAG hydrolyzed from TAG can be phosphorylated to form PA, the precursor of phosphatidylinositol, phosphatidylglycerol and cardiolipin (Fig. 1, pathway 5).

Energy-dense TAG stores can free organisms temporally and spatially from the need for an immediate energy supply and provide a reserve depot that can be used when local resources fail or when specific kinds of fatty acids or lipid precursors are required. In bacteria, the few studies that have been performed suggest that TAG is synthesized during times of stress and resource depletion, and that the TAG is used to generate precursors that will be converted to phospholi-

pids or other products when food supplies improve and growth resumes [4–7]. Similarly, Tetrahymena use their TAG stores primarily for PL synthesis [8]. In animals, TAG is both an energy store (Fig. 1, pathway 3) and a depot of phospholipids and signaling molecules. In this view it is not surprising that higher organisms have developed several pathways for TAG synthesis as well as several modes of regulation. This review will focus primarily on recent studies of the enzymes of TAG synthesis with particular emphasis on cloned isoforms. Excellent reviews are available on TAG synthesis and regulation in plants [9–11] and yeast [12], DAG metabolism [13], CoA-independent transacylases [14,15], and transcriptional control [16]. The older literature contains additional information of interest concerning the enzymes of glycerolipid synthesis [16–19].

2. Enzymes of triacylglycerol synthesis

Studies of the enzymes of glycerolipid biosynthesis date back to the 1950s when most of the pathways were elucidated, largely through work conducted in Eugene Kennedy's laboratory (Fig. 2) [20–22]. Since then, considerable knowledge has been gained about the enzymes that catalyze the lipid biosynthetic reactions and the factors that regulate lipid biosynthesis. Because most of the lipid biosynthetic enzymes are intrinsic membrane proteins, early studies were hampered by difficulties in purifying the enzymes. To compound this difficulty, kinetic analyses were not straightforward since many of the substrates and products are hydrophobic and, thus, insoluble in aqueous solutions. In the last few decades, in part due to advances in technology and the wide availability of nucleotide and amino acid sequences, we have made enormous strides in our understanding of these enzymes at the molecular level. In many cases, sequence information obtained from lipid biosynthetic enzymes of prokaryotes and yeast has provided the means to search the genomic and expressed sequence tag databases for mammalian homologs and most of the genes have now been identified (Table 1).

It has become apparent that multiple isoforms of the enzymes of the lipid synthetic pathway can catalyze the same chemical reaction. In some cases, these isoenzymes are the products of different genes, in others, they are encoded by the same gene but are modified by alternative splicing or post-translational changes. Although people have speculated that each isoenzyme plays a distinct functional role in the pathway of TAG and phospholipid biosynthesis, direct studies are limited. Within the cell, separate lipid pools appear to exist, and these may participate in distinct biological pathways [23,24]. It is also clear that the signaling and modulation of cell function by lipids are important physiological aspects of cellular function. A discussion of these topics however, is beyond the scope of this review. The following sections will instead focus on the de novo biosynthesis of TAG in eukaryotic cells, the isoenzymes that are involved, their subcellular localization, how they are regulated and their putative individual roles in glycerolipid biosynthesis.

3. Synthesis of 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatidate)

The acylation of glycerol-3-phosphate represents the first and committed step in glycerolipid biosynthesis (Fig. 2). The reaction is catalyzed by acyl-CoA: glycerol-*sn*-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15), resulting in the production of 1-acyl-*sn*-glycerol-3-phosphate

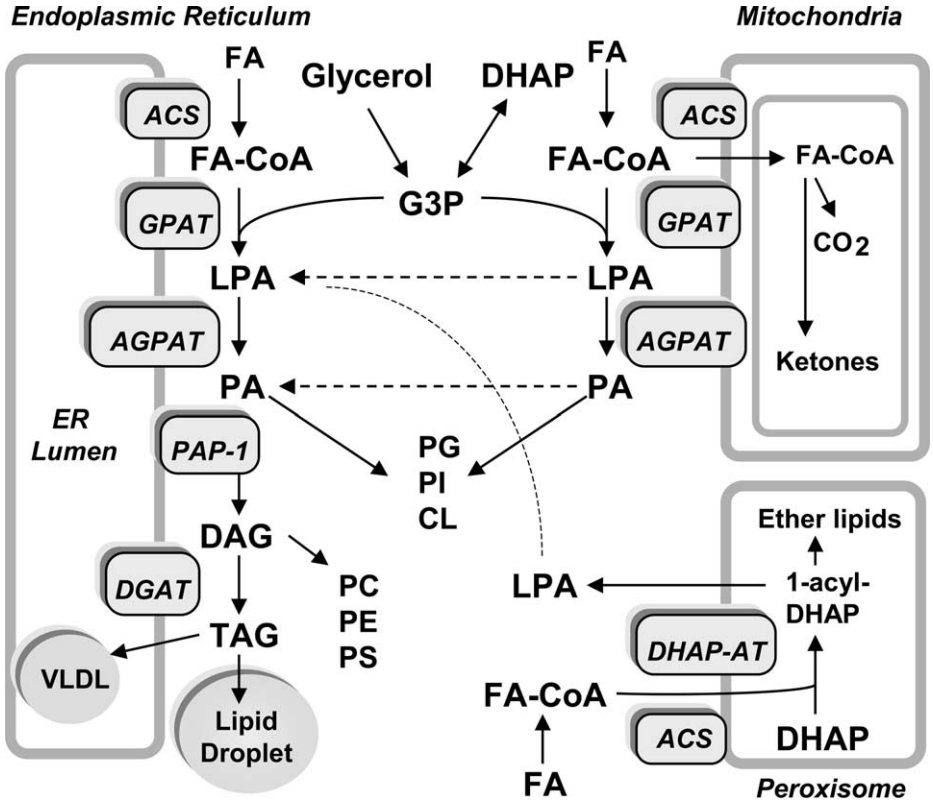

Fig. 2. A organellar view of glycerolipid biosynthesis in liver. LPA and PA can be synthesized in mitochondria but must be transported to the ER where the terminal enzymes for triacylglycerol synthesis are. ACS, acyl-CoA synthetase; AGPAT, acylglycerol-P acyltransferase; CL, cardiolipin; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone-P; DHAP-AT, DHAP acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; G3P, glycerol-3-phosphate; GPAT, glycerol-P acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLC, phospholipase C; PS, phosphatidylserine; TAG, triacylglycerol; VLDL, very low density lipoprotein.

(lysophosphatidate) [25,26]. The glycerol-3-phosphate substrate is produced by the reduction of the glycolytic intermediate dihydroxyacetone-3-phosphate by dihydroxyacetone-3-phosphate dehydrogenase (EC 1.1.1.94), or by the phosphorylation of glycerol by glycerokinase (EC 2.7.1.30). The acyl-CoA substrate is produced by one of several long-chain or very-long-chain acyl-CoA synthetases (EC 6.2.1.3) [27–29]. GPAT exhibits the lowest specific activity of all enzymes in the glycerol-3-phosphate pathway suggesting that this step may be rate limiting [17,30]. Two isoenzymes have been identified based on differences in their pH optima, K_m values, sensitivity to heat and sulfhydryl reagents, and subcellular localization [31].

3.1. Microsomal glycerol 3-phosphate acyltransferase

The microsomal GPAT is sensitive to heating and inhibited by sulfhydryl reagents, and uses a variety of saturated or unsaturated acyl-CoAs [17]. Rat microsomal GPAT has been only par-

Table 1
Enzymes of triacylglycerol synthesis. Accession numbers and chromosomal location

	Accession number		Chromosomal location	
	Mouse	Human	Mouse	Human
GPAT mitochondrial	NP_032175.1	NP_065969	19 52.0 cM	10q25.3
GPAT microsomal	–	–	–	–
DHAP-AT	NP_034452.1	NP_055051.1	8 78.8 cM	1q42.11-42.3
AGPAT-1(α)	NP_061350.2	NP_006402	17	6p21.3
AGPAT-2(β)	NP_080488	NP_006403.2	2	9q34.3
AGPAT-3 (γ)	NP_443747	NP_064517	10 41.8 cM	21q22.3
AGPAT-4 (δ)	NP_080920.2	NP_064518.1	17	6q25.3
AGPAT-5 (ε)	NP_081068	NP_060831	8 7.0 cM	8p23.1
AGPAT-6		XP_067080		Yp11.2
PAP-1 (PPH-1)	–	–	–	–
DGAT-1	NP_034176	NP_036211	15 46.9 cM	8q24.3
DGAT-2	NP_080660	NP_115953	7	11q13.3
MGAT-1	NP_080989	NP_477513	1	2q36.2
MGAT-2	NP_803231	NP_079374	7	11q13.5
MGAT-2^{trunc}	–	–	–	11q13.5
MGAT-3		NP_835470		7q22.1

tially purified [32] and has not been cloned. A putative microsomal GPAT from an oleaginous fungus was purified to homogeneity, but no sequence data has been provided [33]. In most tissues the microsomal GPAT constitutes about 90% of total GPAT activity; in liver, however, it constitutes 50–80% of total activity [17]. Because the specific activity of microsomal GPAT does not vary with dietary or hormonal changes, the enzyme has been thought to function primarily in de novo phospholipid synthesis. However, several studies strongly suggest that the microsomal isoform of GPAT plays a vital role in TAG synthesis as well. For example, in differentiating 3T3-L1 adipocytes, microsomal GPAT specific activity increases 70-fold, while the mitochondrial GPAT (mtGPAT) specific activity increases only 10-fold [34]. In neonatal liver when hepatocyte TAG synthesis increases, microsomal GPAT specific activity increases 74-fold, while mtGPAT increases only 5-fold [35]. And finally, mice deficient in the mitochondrial isoform have diminished, but not absent, fat stores showing that the microsomal GPAT can initiate the formation of at least 50% of the TAG stored in liver and adipose tissue [36].

Microsomal GPAT may be both activated and inhibited by phosphorylation and dephosphorylation. An adipose tissue tyrosine kinase reversibly inactivates the microsomal GPAT [37], but in BC3H-1 myocytes and rat adipocytes, insulin or sodium orthovanadate treatment increases microsomal GPAT activity 1.5- to 2.0-fold [38–40]. NaF blocks the activation of microsomal GPAT activity by insulin, suggesting that activation of microsomal GPAT by insulin is mediated via a phosphatase. The insulin effect may involve a Giα dependant phosphatidylinositol-glycan phospholipase C [41]

3.2. Mitochondrial glycerol 3-phosphate acyltransferase

In contrast to the microsomal GPAT, the mitochondrial GPAT (mtGPAT) is resistant to inactivation by *N*-ethylmaleimide and other sulfhydryl reagents and, in liver, kidney and heart,

has 3- to 10-fold higher activity with 16:0-CoA than 18:1-CoA or other long-chain fatty acyl-CoAs [42–45]. MtGPAT from rat liver was purified to homogeneity [46]. Activity was lost during purification, but could be reconstituted by adding phospholipid, particularly di18:1-phosphatidylglycerol and di18:1-phosphatidylethanolamine. The purified enzyme had activity with 16:0-CoA that was 2 to 3-fold higher than with 18:0-CoA or 18:1-CoA.

Location of mtGPAT on the mitochondrial outer membrane means that its lysophosphatidate product (or the phosphatidate product of mitochondrial AGPAT) must be transported to the endoplasmic reticulum where the terminal enzymes of TAG synthesis and most of the terminal enzymes of glycerophospholipid synthesis are located [17] (Fig. 2). In an in vitro system, liver fatty acid binding protein (FABP) transported lysophosphatidate from its site of synthesis in mitochondria to target microsomes [47,48]. It is not known whether FABP is a major transporter in vivo, however; mice deficient in liver FABP have a normal hepatic content of phospholipid and TAG [49].

3.3. Physiological changes in mtGPAT expression

Mitochondrial GPAT, a protein of 827 aa, has been cloned from mouse and rat liver [50–52]. A cloned rat mtGPAT of 772 aa with a start site at the second methionine was not shown to be active [53]. MtGPAT is expressed in all tissues studied, but in fasted/refed mice, is greatest in liver and adipose tissue, then muscle and kidney, followed by lung and brain [51]. This expression pattern does not correspond to either relative protein levels or to enzyme activity, suggesting a strong influence of post-transcriptional regulation [54]. MtGPAT mRNA increases 30-fold after mice are fasted and then refed a high carbohydrate, fat-free diet; this increase does not occur in mice that were made insulin deficient with streptozotocin [51]. Dibutyryl-cAMP also suppresses the feeding-induced increase in mRNA, suggesting that glucagon caused the low expression observed with fasting. When the recombinant protein with a histidine epitope is expressed in Sf9 insect cells and purified on a Ni2+ matrix column, the inactive protein is best reconstituted with phosphatidylcholine and the reconstituted enzyme prefers 12:0-, 16:0- and 14:0-CoAs compared with unsaturated acyl-CoAs; 20:4-CoA is not a substrate [55].

3.4. mtGPAT functional motifs

Alignment of amino acid sequences from GPAT and other acyltransferases reveals four regions of strong homology that comprise a glycerolipid acyltransferase signature sequence (Table 1) [56–60]. These four motifs are present in GPAT, acyl-glycerol-3-phosphate acyltransferase (AGPAT), dihydroxyacetonephosphate acyltransferase (DHAPAT) and 2-acylglycerophosphatidylethanolamine acyltransferase (LPEAT). It has been proposed that the invariant histidine in Motif 1 acts as a general base to deprotonate the *sn*-1 hydroxyl moiety of glycerol-3-phosphate and facilitate a nucleophilic attack on the acyl-CoA thioester bond [58]. The invariant aspartate residue in Motif 1 may act as part of a charge relay system with the histidine to increase the nucleophilicity of the glycerol-3-phosphate hydroxyl group [56]. Kinetic studies of *Escherichia. coli* with site-directed mutations show that Motifs I and IV function in catalysis and Motifs II and III in glycerol-3-phosphate binding [56]. The GPAT isoforms from *S. cerevisiae* each have a Motif II that does not correspond to the typical FxxR of GPAT isoforms from other species;

Table 2
GPAT (Gpam) family motifs

	Motif I catalysis	**Motif II** G3P binding	**Motif III** G3P binding	**Motif IV** catalysis	**Motif V**
GPAT					
E. coli	303 VPC**H**RSHM**D**YLL	348 GAFFIR**R**TF	382 Y**F**V**EG**G**R**SRTGRLLDPKTGTL	417 ITLI**P**IYI	478 Y**L**NQHVPDWRESI**D**
S. cerevisiae GAT1[a]	77 CAP**H**ANQF**I**DPA	131 GGIPVP**R**IQ	257 I**FP**E**G**GSHDRPSLLPIKAGVA	292 VAVV**P**CGL	
S. cerevisiae GAT2[b]	87 AAP**H**ANQFV**D**PV	134 MAIGVV**R**PQ	249 I**FP**E**G**GSHDRTNLLPLKAGVA	372 KLPL**P**LIV	
Mouse	227 LPV**H**RSHI**D**YLL	272 GGFFIR**R**RL	312 I**FL**E**GTR**SRSGKTSCARAGLL	347 ILVI**P**VGI	
A. thaliana	226 ISN**H**QSEA**D**PAV	254 KCVAGD**R**VI	319 IAPS**GG**RDRPNPSTGEWFPAP	359 GHIY**P**MSL	
DHAPAT human	159 LPS**H**RSYI**D**FLM	204 SGAFFM**R**RT	240 F**FL**E**GTR**SRSAKTLTPKFGLL	275 TYLV**P**ISI	
AGPAT					
E. coli	70 IAN**H**QNNY**D**MVT	112 GNLLID**R**NN	144 M**FP**E**GTR**SRGRGLLPFKTGAF	172 VPII**P**VCV	
S. cerevisiae[c]	102 IAN**H**QSTL**D**IFM	121 GTYFLD**R**SK	153 V**FP**E**GTR**STSELTMLPFKKGA	183 IPIV**P**VVV	
Mouse AGPAT-1	98 VSN**H**QSSL**D**LLG	140 GIIFID**R**KR	172 V**FP**E**GTR**NHNGSMLPFKRGAF	200 VPII**P**IVF	231 V**L**PPVSTEGLTPD**D**
Human AGPAT-1(α)	101 VSN**H**QSSL**D**LLG	143 GVIFID**R**KR	175 V**FP**E**GTR**NHNGSMLPFKRGAF	203 VPIV**P**IVM	233 V**L**PPVPTEGLTPD**D**
Human AGPAT-2(β)	95 VSN**H**QSIL**D**MMG	137 GVFFIN**R**QR	169 IYP**EGTR**NDNGDLLPFKKGAF	197 VPIV**P**VVY	293 V**L**EAIPTSGLTAA**D**
Human AGPAT-3	93 ILN**H**NFEI**D**FLC	140 EIVFCK**R**KW	173 LYC**EGTR**FTETKHRVSMEVAA	202 YHLL**P**RTK	239 S**L**LGILYGKKYEA**D**
Human AGPAT-4	93 VLN**H**KFEI**D**FLG	139 EMVFCS**R**KW	172 IHC**EGTR**FTEKKHEISMQVAR	201 HHLL**P**RTK	238 T**L**LGVLNGKKYHA**D**
Human AGPAT-5	90 LAN**H**QSTV**D**WIV	137 GGIYVK**R**SA	170 I**FP**E**GTR**YNPEQTKVLSASQA	202 HVLT**P**RIK	250 F**L**CKECPKIHIHI**D**
Human AGPAT-6	93 ILN**H**NFEI**D**FLC	140 EIVFCK**R**KW	173 LY**CEGTR**FTETKHRVSMEVAA	202 YHLL**P**RTK	239 S**L**LGILYGKKYEA**D**
LPEAT *E.coli*	33 TPN**H**VSFI**D**GIL	86 AIKHLV**R**LV	102 I**FP**E**GTR**ITTTGSLMKIYDGA	130 ATVI**P**VRI	
WS/DGAT *A. calcoaceticus*	98 SQE**H**STLL**D**RAK	107 ------**R**AK ?	118 EGI**EG**N**R**FAMYFKIHHAMVDG	156 KSIV**P**PWC	

Motifs I-IV were identified and studied for function in *E. coli* [56]. Motif V is deduced from studies of human AGPAT2-related lipodystrophy [61,62]. Bolded amino acids are those that are generally conserved.

[a] YKR067w.
[b] YBL011w.
[c] YDL052c.

Table 3
Amino acids identified as critical for acyltransferase function

	Motif I catalysis	**Motif II** G3P binding	**Motif III** G3P binding	**Motif IV** catalysis	**Motif V**
E. coli GPAT	303 VPC**H**RSHM**D**YLL	348 G**A**F**FI**R**R**TF	382 Y**F**V**EG**GRSRTGRLLDPKTGTLSM	417 ITLI**P**IYIGY	
Mouse GPAT	227 LPVHRSHIDYLL	271 GGFFIR**R**RL	312 LEI**F**L**E**GT**R**SRSGKTSCARAGLL	347 ILVIPVGISY	
S. cerevisiae GAT1	77 CAPHANQFIDPA	131 GGIPVPRIQ	255 VGIFPEG**G**SHDRPSLLPIKAGVA	292 VAVVPCGLHY	
Human AGPAT-2	95 VSNHQSILDMMG	136 **G**GVFFINRQR	169 IYP**E**GTRNDNGDLLPFKKGAFYL	197 VPIVPVVYSS	293 V**L**EAIPTSGLT**AA**D
Human DHAPAT	159 LPSHRSYIDFLM	204 SGAFFMR**R**T	240 FFLEGTRSRSAKTLTPKFGLLNI	275 TYLVPISISY	
Human Tafazzin	66 VSN**H**QSCMDDPH	86 IWNLKLM**R**WT	177 I**F**P**E**GK**V**NMSSEFLRFKWGI**G**RL	206 NP**IL**P**L**WHV**G**	237 VLI**G**KPFSALPVLE

The underlined and bolded amino acids are critical for function as shown by site-directed mutagenesis and/or mutational analysis in *E. coli* [56,58,59], yeast [81], and mouse GPAT [70] and by mutational analysis of the human genetic disorders, AGPAT2-related lipodystrophy [61,62], DHAPAT deficiency causing rhizomelic chondrodysplasia punctata type 2 [63], and tafazzin deficiency causing Barth syndrome [64–68].

thus, these Motif II sequences (Table 2) require verification by site-directed mutagenesis. Analysis of amino acid mutations in human genetic disorders that involve acyltransferases, AGPAT-2-related lipodystrophy [61,62], rhizomelic chondrodysplasia punctata type 2 [63], and Barth syndrome/tafazzin [64–68], indicates that most amino acid substitutions that cause disease are in Motifs I-IV (Table 3). However, additional mutations in AGPAT-2 deficiency and Barth syndrome indicate an important 5th motif that was not previously discerned. How Motif V contributes the function of these acyltransferases is as yet unclear.

Hydrophobicity analysis of mitochondrial GPAT predicts two transmembrane domains, and protease digestion and immunolocalization of epitope tags of recombinant mtGPAT indicate that the N and C termini are orientated toward the cytosol and that an internal loop of the protein is exposed to the intermembrane space [69]. Although the active site (Motifs I-IV) is located within the N-terminal domain, modifying the internal loop results in loss of activity, suggesting that this region plays a critical structural or regulatory role.

3.5. Regulation of mtGPAT

The mtGPAT is regulated nutritionally and hormonally in a manner consistent with its having an important role in TAG synthesis [70,71]. Both gene transcription and protein modification regulate mtGPAT activity. Transcription of mtGPAT is enhanced markedly during adipocyte differentiation [72] via the transcription factors NF-Y and the sterol regulatory element binding protein-1 (SREBP-1/ADD1) [72,73]. Consistent with studies on the effects of diet on mtGPAT activity, the promoter region also responds to carbohydrate. When 3T3-L1 adipocytes are incubated in medium containing 5 and 25 mM glucose, luciferase reporter activity under control of the mtGPAT promoter increases 3- and 8-fold, respectively [74]. Conversely, fasting reduces hepatic mtGPAT protein expression and activity, and refeeding a high carbohydrate or a high sucrose diet increases both [54,72]. In streptozotocin-diabetic mice, mtGPAT expression and activity is drastically reduced, and insulin increases mRNA levels about 20-fold after 6 h [51]. The mtGPAT mRNA has long 5′ and 3′ untranslated regions that probably contribute to its regulation.

The importance of mtGPAT in TAG synthesis has been confirmed by studies of over-expressed GPAT in cells and in mtGPAT null mice. When mtGPAT is stably over-expressed 3.8-fold in Chinese hamster ovary cells, the TAG content is 2.7-fold higher than in vector-control cells, and the incorporation of [^{14}C]oleate into TAG is 3-fold higher [75], whereas incorporation of [^{14}C]oleate into phosphatidylcholine decreases 30% [75]. Mice lacking mtGPAT weigh less than wild type mice, have 30% lighter gonadal fat pads, a 40% decrease in liver TAG content, and reduced plasma TAG and VLDL–TAG secretion [36]. In mtGPAT$^{-/-}$ liver, phosphatidylcholine and phosphatidylethanolamine contain 21% less 16:0 in the *sn*-1 position, as would be predicted because of the preference of mtGPAT for 16:0-CoA. Surprisingly, however, both phospholipids contain 38% more 20:4 in the *sn*-2 position, thereby showing that the fatty acid in the *sn*-1 position influences remodeling by phospholipid acyltransferases and that mtGPAT indirectly controls how much 20:4 is esterified at the *sn*-2 position. The GPAT$^{-/-}$ mice also show that mtGPAT is critical, but not essential for hepatic TAG synthesis and VLDL secretion [36], and that it plays a major role in regulating the fatty acid composition of glycerophospholipids.

In addition to transcriptional regulation, mtGPAT is regulated via dephosphorylation and phosphorylation. The enzyme contains several putative casein kinase sites, and incubation of rat

liver mitochondria with casein kinase II results in a 2-fold activation of mtGPAT activity, though phosphorylation of the protein was not shown directly [76]. MtGPAT is also regulated by AMP-activated kinase (AMPK), a sensor of cellular energy supply [77]. AMPK is activated when cellular ATP levels are relatively depleted and AMP levels rise. AMPK stimulates fatty acid oxidation and inhibits several synthetic pathways including those of cholesterol, glycogen and fatty acids [77]. Incubating hepatocytes with an activator of AMPK, 5-amino-4-imidazolecarboxamide riboside (AICAR), inhibits the incorporation of radiolabeled fatty acid or glycerol into TAG or phospholipids [78]. When mouse soleus muscle is treated with AICAR, TAG biosynthesis is reduced, and $^{14}CO2$ production increases [78]. Purified recombinant AMPK inhibits mtGPAT directly in a time and ATP-dependant manner [78], and increases phosphorylation of recombinant mtGPAT.[1] AMPK minimally affects microsomal GPAT, and has no effect on diacylglycerol acyltransferase or acyl-CoA synthetase activities, suggesting that the effect of AMPK on TAG biosynthesis occurs primarily via mtGPAT. When rats exercise on a treadmill, mtGPAT activity decreases 50% in liver and adipose tissue but not muscle. The activity decrease is thought to be mediated by AMPK because AMPK activity concomitantly increases, and because adding AICAR to hepatocyte membrane preparations results in similar effects on mtGPAT [79]. Taken as a whole, these data suggest that the action of AMPK reciprocally regulates acyl-CoA channeling towards β-oxidation and away from glycerolipid biosynthesis. AMPK phosphorylates and down-regulates acetyl-CoA carboxylase, thereby decreasing the production of malonyl-CoA, a strong inhibitor of carnitine palmitoyltransferase-1. Without inhibition by malonyl-CoA, the activity of carnitine palmitoyltransferase-1, the rate-controlling step in β-oxidation, increases, as does fatty acid oxidation. Thus, when cellular fuel supplies are low, AMPK increases the flux of acyl-CoA into the pathway of β-oxidation while simultaneously inhibiting mtGPAT activity and TAG synthesis.

A second possible reason for the lower body weight observed in mtGPAT$^{-/-}$ mice is a decrease in the synthesis of specific mitochondrial phospholipids like cardiolipin.[2] Cardiolipin is the major phospholipid of the inner mitochondrial membrane and is critical for the function of several proteins, including cytochrome oxidase; when cardiolipin is deficient, oxidative phosphorylation is diminished [80]. Thus, the lower body weight of mtGPAT$^{-/-}$ mice could be due either to impaired mitochondrial respiration or, because mtGPAT lies at the mitochondrial outer membrane, to lack competition for acyl-CoAs which are then diverted towards β-oxidation.

3.6. Yeast and plant GPAT

In yeast, two GPAT genes, GAT1 and GAT2 (originally identified as a choline transporter suppressor designated SCT1) are encoded by separate genes. The amino acid sequences of the encoded enzymes share homology with *E. coli* GPAT (PlsB) and mammalian mitochondrial GPAT, especially at Motifs I, III, and IV (Table 2) [81]. When the yeast enzymes are overexpressed, they can use both glycerol-3-phosphate and dihydroxyacetone phosphate as substrates [81]. Gat1p has a broad acyl-CoA specificity similar to that of rat microsomal GPAT and can use 16:0-, 18:0-, 16:1- and 18:1-CoAs. The acyl-CoA specificity of Gat2p, however, exhibits a 2.5- to

[1] Lewin, TM, Coleman, RA. Unpublished data.
[2] Hammond, LM, Coleman, RA. Unpublished data.

5-fold preference for 16 carbon acyl-CoAs compared to 18 carbon acyl-CoAs [81]. Inactivating either GAT alone does not affect yeast growth, but growth ceases when both genes are inactivated. Metabolic studies show that TAG biosynthesis increases 50% in *gat1*Δ yeast and decreases by 50% in *gat2*Δ yeast, indicating that Gat2p initiates the major route for TAG biosynthesis [82]. The *gat1*Δ and *gat2*Δ yeast also have alterations in phosphatidylcholine synthesized via the CDP-choline pathway [82].

Plants contain three types of GPAT, a membrane-bound enzyme located in mitochondria, and soluble proteins located in cytosol and chloroplasts [83]. Only the chloroplast isoforms have been cloned. These contain glycerolipid acyltransferase signature sequences that differ somewhat from the mammalian GPAT and AGPAT domains (Compare the motifs in Table 2 for *A. Thaliana* (Accession number Q43307).

3.7. Dihydroxyacetone phosphate acyltransferase pathway

Although 93% of TAG is produced via the glycerol-3-phosphate pathway under normal physiological conditions in liver [84], lysophosphatidate can also be formed by the acylation of dihydroxyacetone-phosphate by acyl-CoA:DHAP acyltransferase (DHAP-AT) (EC 2.3.1.42) followed by reduction of the newly formed 1-acyl-DHAP to lysophosphatidate by DHAP oxidoreductase (Fig. 1). The contribution of the DHAP-AT is not clearly understood and may depend on the cell system investigated and the experimental conditions. In 3T3-L1 adipocytes, for example, it was reported that 40–50% of the TAG synthesized is derived from glucose via the DHAP pathway [85]. This surprisingly large contribution of the DHAP pathway to TAG biosynthesis could have occurred because the cells had been pre-incubated overnight with media that lacked glucose. Glucose (and insulin) deprivation would be likely to severely diminish mtGPAT mRNA in adipocytes [50,51], thereby increasing the relative metabolic flux through the DHAP pathway. Consistent with the interpretation that the DHAP pathway does not play a major role in TAG synthesis is the observation that the required acyl/alkyl-DHAP reductase (EC 1.1.1.101) specific activity increases only 3- to 5-fold during adipocyte differentiation [34]. CHO cells deficient in this activity decrease the incorporation of ^{32}Pi into diacyl-phospholipids, indicating that the DHAP pathway may contribute substantially to glycerolipid synthesis in some cell types [86]. Possibly the DHAP pathway contributes significantly to hepatic TAG synthesis in untreated type 1 diabetes when mtGPAT expression is very low. Older studies (summarized in detail in [87]), variably suggest that the DHAP pathway does or does not make a significant contribution to diacyl-glycerolipid synthesis.

DHAP-AT activity is present in both microsomes and peroxisomes. DHAP-AT activity in peroxisomes is required for plasmalogen biosynthesis [88]; the microsomal enzyme activity is believed to be a second function of the microsomal GPAT because the activities have identical pH profiles, thermolability, and sensitivity to various inhibitors, and their glycerol-3-phosphate and DHAP substrates are mutually competitive [34,88–90]. *Saccharomyces cerevisiae* GPAT mutants are equally defective in DHAP-AT activity, thereby establishing the genetic identity of the two activities in yeast [91]. 1-Acyl-DHAP reductase (EC 1.1.1.101), which catalyzes the formation of lysophosphatidate, is enriched in peroxisomes but is also found in microsomes [92].

Human and mouse DHAP-AT have been cloned. DHAP-AT encodes a protein of about 77 kDa which contains a PTS1 (alanine-lysine-leucine) motif at the C-terminus for peroxisomal targeting [63,93]. Mouse DHAP-AT cDNA shares 80% identity with the human DHAP-AT cDNA and is

highly expressed in heart, liver, and testis, with lower expression in brain, lung, skeletal muscle and kidney [93]. DHAP-AT is deficient in patients with rhizomelic chondrodysplasia punctata, a peroxisomal disorder, which results in severe growth impairment and mental retardation, congenital contractures, dysmorphic facial appearance and rhizomelic shortening of the upper extremities [63]. Analysis of the DHAP-AT from these patients indicates that all have a mutation at Arg211 in Motif II (Table 3). No disturbance in TAG has been reported in these patients.

4. Synthesis of phosphatidate

Phosphatidate is synthesized de novo from the acylation of lysophosphatidate in a reaction catalyzed by acyl-CoA:1-acylglycerol-*sn*-3-phosphate acyltransferase (AGPAT) (also called lysophosphatidate acyltransferase or LPAAT) (EC 2.3.1.51). Activity is located in both mitochondria and microsomes, and enzyme activity has been detected in the plasma membrane [94].

4.1. *Acylglycerol*-sn-*3-phosphate acyltransferase 1*

Human AGPAT-1, which encodes a 283 aa protein was cloned based on homology with yeast, E. coli, and coconut AGPATs [95,96]. Mouse AGPAT-1 has also been cloned [97]. Alignment of AGPAT-1 from multiple species identifies the four homology motifs present in mtGPAT (Table 2). Programs predict two signal peptides and two transmembrane domains (aa 130-147 and 195-211) with the N and C termini in the lumen of the ER and the active site motifs facing the cytosol [98]. An alternative prediction suggests a single transmembrane domain [99]. AGPAT-1 mRNA is detected in all human tissues with high expression in liver, heart, immune cells, and epithelium [95,96,100]. The role of AGPAT in signal transduction cannot be excluded because interleukin-1 activates AGPAT in human mesangial cells, resulting in increases in phosphatidic acid with a fatty acid composition that differs from that of major membrane phospholipids [94]. Over-expression of AGPAT-1 or AGPAT-2 in A549 and ECV304 cells correlates with an enhanced TNF-α and IL6 release induced by interleukin-1β [95]. In addition, the AGPAT-1 gene has been mapped to the class III region of the human major histocompatibility complex [98]. When AGPAT-1 is stably overexpressed in C2C12 myocytes or 3T3-L1 fibroblasts (later differentiated to adipocytes), total AGPAT activity increases 20–25%. Overexpression of AGPAT-1 in adipocytes increases [3H]oleate incorporation into TAG 2-fold, and in C2C12 cells [^{14}C]glucose incorporation into total lipid increases 33% [101]. Surprisingly however, the overexpressed protein is present in cytosol, rather than membranes, so this study may not be conclusive regarding the physiological function of AGPAT-1 [101]. When AGPAT-1 is stably expressed in CHO cells, an ER-staining pattern is seen [98] and no immunostaining of the plasma membrane is observed. AGPAT-1 can also synthesize acyl-CoAs and LPA via a reverse reaction [102].

AGPAT-1 over-expressed in baculovirus uses both saturated and unsaturated acyl-CoAs of 12-18 carbons [98]. Highest activity occurs with 14:0-, 16:0-, and 16:1-CoAs and intermediate activity with 18:1- and 20:4-CoAs. These results suggest either that other AGPAT isoforms or glycero-

lipid remodeling are responsible for the usual *sn*-2 enrichment of 20:4. Of interest is the alteration in fatty acid substrate specificity that occurs with site-directed changes of threonine 122 in *E. coli* AGPAT [103]. Substitution of alanine increases the in vitro specificity for 18:1-CoA and 18:2-CoA, whereas substitution of leucine increases substrate specificity for 24:0-CoA.

4.2. *Acylglycerol*-sn-*3-phosphate acyltransferase 2*

Human AGPAT-2, which encodes a 278 amino acid protein, was cloned by homology to yeast AGPAT [95]. Although it was originally predicted that AGPAT-2 had four transmembrane domains, this prediction would situate Motif I (NHQSxxD) between the 2nd and 3rd predicted transmembrane domains, and Motif III (PEGTR) between the 3rd and 4th transmembrane domains (Table 2) [99,100]. Since these motifs are essential for catalysis, they are unlikely to lie on opposite sides of a membrane, and the predicted topography must be incorrect. AGPAT-2 has 35% amino acid identity with human AGPAT-1 [95]. Its mRNA is expressed in most human tissues with highest expression in heart and liver [95,100]. The expressed recombinant protein exhibits higher activity towards 20:4-CoA than 18:0- or 16:0-CoAs [100]. AGPAT-2 is deficient in the Berardinelli-Siep form of congenital lipodystrophy, indicating that the other AGPAT isoforms cannot compensate for its loss and that AGPAT-2 is critical for TAG synthesis in adipose tissue where its expression is 2-fold greater than AGPAT-1 and where AGPATs-3, -4, and -5 are barely detectable [61]. An alternate possibility is that an increase in the LPA substrate or a decrease in the phosphatidate product alters a critical signaling event required for adipocyte differentiation or function. Because both AGPAT-1 and-2 are present in adipocyte endoplasmic reticulum membranes [98], their phosphatidate products must lie in separate pools, only one of which can be used for TAG synthesis.

4.3. *Acylglycerol*-sn-*3-phosphate acyltransferase 3–6*

Four other human nucleotide sequences encoding putative AGPATs have not yet been studied (Table 1). A bioinformatics analysis predicts that AGPAT-3 and-4 are ER membrane proteins with signal peptides (which may not be cleaved) and that 3 transmembrane helices are present that place Motifs I and III of the active site (Table 2) on opposite sides of the membrane [104]. It is more likely that one or more of the putative transmembrane domains is actually a hydrophobic domain that interacts with the membrane but does not cross it. A study of conserved sequences identifies a sixth family member that is similar to AGPAT-3 and-4 (Table 1).

4.4. *Alternative routes for phosphatidate synthesis*

Phosphatidate can also be synthesized from diacylglycerol by diacylglycerol kinase (EC 2.7.1.107). This reaction is believed to generate a phosphatidate that is a signaling molecule rather than a precursor for TAG biosynthesis [105]. Additional genes that are not members of the AGPAT family encode cytosolic activities that acylate lysophosphatidic acid. These gene products are called endophilin and CtBP/BARS, and act during vesicle fusion and recycling [106–109]. These acyltransferases may convert lysophosphatidic acid to the bulkier phosphatidate on

one leaflet of a bilayer, thereby changing the relative areas of the inner and outer leaflets, enhancing the curvature of the membrane, and enabling vesicles to form.

5. Synthesis of diacylglycerol

The phosphatidate formed by AGPAT occupies a central branch point in lipid biosynthetic pathways [26]. It can be converted to CDP-diacylglycerol, which serves as the precursor for the biosynthesis of the acidic phospholipids such as phosphatidylinositol, phosphatidylglycerol and cardiolipin (Fig. 1) [110]. Alternatively, phosphatidate can be dephosphorylated to produce diacylglycerol, the precursor of TAG, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine.

5.1. Phosphatidic acid phosphatase-1

Diacylglycerol is produced from phosphatidate in a reaction catalyzed by phosphatidic acid phosphatase (PAP) (EC 3.1.3.4). At least two types of PAP exist in mammalian tissues [111], but only PAP-1 is involved in TAG and phospholipid synthesis. PAP-1 is Mg^{2+}-dependent and inactivated by *N*-ethylmaleimide [111, 112], and stimulation by fatty acids or acyl-CoAs causes the enzyme to translocate from the cytosol to the endoplasmic reticulum [113]. PAP-1 is displaced from the membrane by okadaic acid, suggesting that its location may be regulated by a protein kinase [113]. Both regulation and subcellular localization suggest that PAP-1 provides DAG for TAG and phospholipid biosynthesis [113]. PAP-1 activity is also stimulated by glucagon, glucocorticoids, cAMP, and growth hormone and is inhibited by insulin. These responses, some of which are opposite what might be expected for an enzyme that promotes TAG synthesis, are hypothesized to be part of a response that protects the liver from stress-mediated influxes of fatty acids [114]. In recent years PAP-1 has received little attention; the protein has not been purified, and the cDNA has not been cloned.

5.2. Phosphatidic acid phosphatase-2

The reaction catalyzed by PAP-2 produces diacylglycerol from phosphatidic acid released from membrane phospholipid by phospholipase D during signal transduction pathways [115–117]. PAP-2 is a six-transmembrane-domain integral protein localized to the plasma membrane, that does not require Mg^{2+} for activity, and is not inactivated by *N*-ethylmaleimide [116,118]. In addition to hydrolyzing phosphatidic acid and lysophosphatidic acid, PAP-2 degrades sphingosine-1-phosphate and ceramide-1-phosphate and has been variously named lipid phosphate phosphohydrolase (LPP) and phosphatidate phosphohydrolase (PPH). Although the PAP-2 pathway does not comprise a quantitatively important route for glycerolipid synthesis, diacylglycerol that has been produced at the plasma membrane can move to the endoplasmic reticulum where it is a substrate for TAG and phospholipid synthesis [119,120].

5.3. Monoacylglycerol acyltransferase pathway

The monoacylglycerol pathway of diacylglycerol synthesis forms a major route in intestinal enterocytes and may be important in other tissues. After pancreatic lipase preferentially hydrolyzes the *sn*-1 and *sn*-3 ester bonds of dietary TAG, the resulting *sn*-2-monoacylglycerol and fatty acid products enter enterocytes. Monoacylglycerol can also be synthesized by the acylation of glycerol. A microsomal acyl-CoA: glycerol acyltransferase activity was measured in bovine heart, liver, kidney, skeletal muscle and brain [121]. The specific activity was highest with 20:4-CoA, but the importance of glycerol acyltransferase in monoacylglycerol synthesis may be limited by its high apparent K_m for its glycerol substrate (1.1 mM). Hyperglycerolemic conditions, such as certain forms of muscular dystrophy, diabetes, fasting, extreme cold, and cardiac ischemia may potentiate the direct acylation of glycerol. Glycerol acyltransferase has not yet been purified or cloned.

Monoacylglycerol is converted to diacylglycerol in a reaction catalyzed by acyl-CoA:monoacylglycerol acyltransferase (MGAT) (EC 2.3.1.22) [122]. This initial step in the synthesis of TAG secreted in chylomicra is critical for dietary fat absorption. It has been estimated that after a meal 75% of the TAG synthesis in the small intestine occurs via the monoacylglycerol pathway [123]. Partial purification of MGAT from rat intestine identified a protein of 37 kDa [124]. MGAT has also been studied in adipose tissue [125], cardiomyocytes [126], and liver [127,128] where it may preserve polyunsaturated fatty acids at the *sn*-2 position during periods of TAG hydrolysis and resynthesis [125,129]. MGAT specific activity is greatest in small intestine with much lower activity observed in stomach, kidney, brown and white adipose tissues, liver, testis, brain, skeletal muscle, and heart [130]. Very high MGAT activity that coincides with the suckling period has also been studied in neonatal rat liver [128,131]. Based on substrate preferences and enzyme characterization, it was hypothesized that the MGAT activities in intestinal mucosa and neonatal liver were separate isoenzymes [132,133]. It now appears that this conclusion was an underestimate. At least three genes encode MGAT; these were first identified as members of the DGAT-2 family [134], and have been designated MGAT-1, -2 and -3. The isoenzymes are all believed to be present in the endoplasmic reticulum [130,135–137]. MGAT-1 and -2 acylate both *sn*-2- and *sn*-1(3)-monoacylglycerols [130,135,137], but MGAT-3 is more highly specific for *sn*-2-monoacylglcyerol [136]. MGAT-1 mRNA is expressed in many mouse tissues, but not in intestine, whereas MGAT-2 and -3 are primarily expressed in the small intestine, suggesting that they are the main contributors to fat absorption. In light of studies in human fetal small intestine showing the first appearance of MGAT activity at 12 weeks [138], it would be interesting to know which of the three MGATs is responsible. At present, studies are lacking concerning the regulation of the three cloned MGAT isoforms, and one wonders whether each has a distinct, overlapping, or interacting function.

Neonatal liver MGAT was partially purified [139] and studied in mixed micellar assays which allow one to determine the stoichiometry and specificity of lipid requirements of intrinsic membrane proteins [18,140]. Phosphatidic acid, lysophosphatidic acid, and anionic phospholipids were potent activators, and *sn*-1,2-dioleoylglycerol, but not the 1,3-diacyl- or the alkylglycerol analogs, was a weaker but additive activator [141]. Sphingosine and sphinganine were a strong inhibitors of MGAT activity in both mixed micelles and in cultured neonatal rat hepatocytes [142], and MGAT kinetics were altered by fatty acids and anionic

phospholipids [143]. These data support the hypothesis that regulatory links are present between the synthesis of complex lipids via the monoacylglycerol pathway and lipid second messengers, the influx of fatty acids from high-fat diets, and the influx of fatty acids from the hydrolysis of adipocyte TAG during diabetes or fasting. The neonatal and intestinal mucosa MGAT activities were shown to differ. Whereas the liver activity was highly specific for *sn*-2-monoacylglycerol, the intestinal activity acylated *sn*-1-monoacylglycerol and *sn*-1- and 2-monoalkylglycerols at rates up to 78% of 2-monoacylglycerol, the preferred substrate [132,133]. The neonatal liver and intestinal activities also differed in thermolability, and inactivation with diethylpyrocarbonate and trinitrobenzene, indicating differently accessible critical histidine and lysine residues.

5.4. Monoacylglycerol acyltransferase-1

Mouse MGAT-1, identified by its sequence homology to acyl-CoA:diacylglycerol acyltransferase-2 (DGAT-2) [130], is a predicted 335 aa protein with at least one transmembrane domain near the amino terminus. MGAT-1 overexpressed in Sf9 insect cells acylates all stereoisomers of monoacylglycerol equally and uses a variety of acyl-CoAs. Highest activity is measured using 20:4-CoA, followed by 18:1- and 18:2-CoAs. Expression in COS-7 cells shows an immunocytochemistry pattern consistent with ER staining. In mouse tissues mRNA expression is observed in stomach, kidney, uterus, liver, and white and brown adipose tissues.

5.5. Monoacylglycerol acyltransferase-2

MGAT-2 was cloned based on sequence homology to the mouse MGAT-1 and DGAT-2 [134,135]. MGAT-2 is 52% identical to MGAT-1, and is predicted to have 334 aa and to contain at least one transmembrane domain near the N-terminus [137]. Highest mRNA expression occurs in the human liver, small intestine, stomach, kidney, colon and white adipose tissue. Mouse MGAT-2 has been variably reported to be expressed only in small intestine [137] or primarily in small intestine but also in kidney, adipose, stomach, liver, skeletal muscle and spleen [135]. Expression of MGAT-2 in COS-7 cells showed immunocytochemistry staining consistent with ER and possibly Golgi, although specific subcellular markers were not used [137]. Overexpression of human MGAT-2 in Sf9 insect cells and in mammalian cells results in marked increases in MGAT activity, as well as a low DGAT activity [135,137,144]. Although human MGAT-2 expressed in insect cell membranes uses both *sn*-2 and *sn*-1 monoacylglycerols, activity was about 3 times higher with the *sn*-2 substrates. The recombinant enzyme is variably described as showing little [137] or great [144] preference for unsaturated monoacylglycerols of 10–20 carbons. The enzyme esterifies long-chain saturated acyl-CoA substrates poorly [137]. A truncated form of human MGAT-2 ($hMGAT^{trunc}$) appears to occur through nonsplicing of coding exons 4 and 5, causing a premature stop codon. This gene product lacks MGAT activity, but is hypothesized to have some function because it is highly expressed in stomach [137].

5.6. Monoacylglycerol acyltransferase-3

Human MGAT-3 was cloned based on similarity to DGAT-2, and has 49% identity to DGAT-2, 44% identity to MGAT-1, and 46% identity to MGAT-2 [136]. Overexpression of MGAT-3 in Sf9 insect cells shows that MGAT-3 encodes for a 36 kDa protein which is predicted to have as many as five transmembrane domains. Expression in COS-7 cells co-localizes with an endoplasmic reticulum marker. Human MGAT-3 is highly expressed in the gastrointestinal tract, particularly in the ileum, but with 3- to 4-fold lower expression in duodenum, jejunum, caecum, colon, rectum, and liver. The high expression in ileum is surprising because dietary lipid is almost completely absorbed in the duodenum and jejunum [123] unless fat intake is very high [145]. MGAT-3 expressed in Sf9 insect cells prefers *sn*-2-monoacylglycerol and uses a broad range of acyl-CoAs, but highest activity is observed with 16:0- and 18:1-CoA.

6. Synthesis of triacylglycerol

Diacylglycerol lies at the branchpoint between phosphatidylcholine, phosphatidylethanolamine, and TAG synthesis. The regulation of DAG flux is strongly influenced by CTP:phosphocholine cytidylyltransferase activity and the requirement for phosphatidylcholine synthesis [146]. The formation of TAG can be catalyzed by several different activities, including diacylglycerol:acyl-CoA acyltransferase (DGAT) (EC 2.3.1.20) which acylates *sn*-1,2-DAG (or 2,3-DAG) [147], *sn*-1,2(2,3)-diacylglycerol transacylase [148], wax ester/DGAT [149], or lecithin-DAG transacylase [150]. Of these enzymes, only DGAT and DAG transacylase have been measured in mammalian sources. DGAT activity is highest in organs that have high rates of TAG synthesis: adipose tisue, liver, lactating mammary gland, small intestinal mucosa, and adrenal gland [151]. Older studies suggesting that DGAT activity is regulated acutely by phosphorylation-dephosphorylation mechanisms were summarized recently [71].

6.1. Diacylglycerol acyltransferase-1

DGAT-1 was cloned through its homology to acyl-CoA:cholesterol acyltransferase (ACAT). Mouse DGAT-1 has 20% identity to mouse ACAT-1 [152]. The predicted protein of 498 aa contains the same conserved serine that is required for ACAT activity [152] and its topography is likely to be similar to ACAT-1 which has seven transmembrane domains [153]. The conserved FYxDWWN motif in DGAT-1 and ACAT family members may be required for acyl-CoA binding [154]. Both ACAT-1 [155] and DGAT-1 [156] are homotetramers.

When its cDNA is expressed in H5 insect cell membranes, DGAT-1 activity is dependent on added acyl-CoA, and diacylglycerol is the only acyl-acceptor identified. DGAT-1 mRNA expression in humans is highest in adipose tissue and small intestine with lower abundance in heart, skeletal muscle, thymus, and testis, but is present in every adult tissue examined as well fetal brain, lung, liver, and kidney [134,152]. In yeast, lecithin cholesterol acyltransferase (LRO1) provides the major pathway for TAG synthesis [150], but the acyl-CoA sterol acyltransferase-2 (ARE2) and DGA1 genes encode minor pathways [157,158]. However, when all three genes are

deleted, yeast growth is minimally impaired, indicating that TAG synthesis is not essential in yeast under normal laboratory growth conditions [157].

Surprisingly few studies have examined DGAT regulation. Specific activity increases 60-fold in differentiating 3T3-L1 adipocytes [30], but mRNA expression of DGAT-1 [152] and DGAT-2 [134] increase only 8- and 30-fold, respectively, suggesting the possibility of posttranscriptional regulation. In both 3T3-L1 fibroblasts and adipocytes, over-expression of DGAT-1 by an adenovirus construct increases mRNA >20-fold but, in each case, DGAT protein increases 2-fold at most [159]. Protein stability is not altered, and steady state levels of the DGAT-1 protein remain constant even when proteolysis is blocked. Despite older data consistent with inactivation by an ATP-dependent tyrosine kinase [37], mutation of a conserved tyrosine (Y316F) yields a fully active enzyme. These data, taken as a whole, suggest that DGAT is regulated translationally [159]. In neonatal liver, it has not been reported which isoform is responsible for the 3.4-fold increase in specific activity [35]. Omega-3 fatty acids reduce TAG synthesis and secretion, perhaps, in part, because the CoA ester of eicosapentanoic acid either inhibits DGAT activity or competes with other acyl-CoAs [160].

DGAT-$1^{-/-}$ mice (50%C57BL/6J and 50% 129/SvJae) have normal weight on chow, but resist weight gain on a diet that is 21% fat, and have fat pads that are smaller than those of wild type mice [161]. Backcrossed animals eat more [162] and their hyperphagia increases during exposure to cold, suggesting that intake compensates for the increased metabolic rate [163]. Indirect calorimetry (performed with the F2 generation) showed that the DGAT-$1^{-/-}$ mice have an increased metabolic rate due, in part, to an increase in activity. Surprisingly, female DGAT-$1^{-/-}$ mice are unable to produce milk; their mammary glands lack lipid droplets in epithelial cells although the surrounding adipocytes appear normal [161]. In back-crossed BL/6J DGAT-$1^{-/-}$ mice, adipose and skeletal muscle lipid accumulation is reduced 30–40%, and knockout mice require a 20% greater glucose infusion rate in hyperinsulinemic-euglycemic clamp studies, showing that insulin sensitivity is increased [162]. The brown adipose tissue from the DGAT-1 knockout mice expresses more UCP1, and white adipose tissue expresses increased amounts of UCP3, PPARα, and acyl-CoA oxidase and decreased amounts of PPARγ, fatty acid synthase, and leptin, similar to mice overexpressing leptin [162]. Because both leptin levels and these leptin target genes are reduced in the DGAT-1 knockout mice, it was suggested that the DGAT-$1^{-/-}$ animals have increased leptin sensitivity [162]. The importance of the endocrine function of white adipose tissue to whole body metabolism is inferred by transplantation of DGAT-$1^{-/-}$ white adipose tissue into wild type mice [164]. The weight gain of the transplanted wild type mice is lower on a high-fat diet (21% fat), and insulin sensitivity increases. DGAT-1 deficiency in fat cells appears to increase the expression of adiponectin which acts to resist obesity and increase glucose uptake [164]. Although DGAT-1 does not appear to be required for TAG absorption when mice are fed chow, with an acute high-fat challenge, only the wild-type mice increased plasma TAG, showing that even though DGAT-2 and diacylglycerol transacylase are still present in the small intestine, the absence of DGAT-1 limits the formation of TAG-rich chylomicra [165].

Several crosses of DGAT-$1^{-/-}$ mice with other models of obesity have proven informative. DGAT-$1^{-/-}$ mice crossed with A^y/a mice protect the mice against obesity and reduce plasma insulin levels 80%, perhaps by increasing insulin sensitivity [162]. Although crosses with *ob*/*ob* mice do not lessen the obesity, DGAT-2 expression in white adipose tissue is upregulated about

3-fold, suggesting that leptin (which is present in Ay/a obese mice) would normally down-regulate DGAT-2 [162]. The atrophic sebaceous glands and abnormal fur of DGAT-1$^{-/-}$ mice are absent in crosses of DGAT-1$^{-/-}$ and *ob*/*ob*, suggesting that leptin modifies skin and fur physiology [166].

When DGAT-1 is over-expressed 2-fold only in WAT (transgenic aP2-DGAT-1$^{-/-}$ mice), the adipocytes are enlarged and fat pad weights are greater than in wild type controls [167]. With a high-fat diet, these mice become even more obese but, unlike other models of excess adiposity, obesity is confined to the white adipose tissue and the mice do not develop increased TAG stores in liver and skeletal muscle, or insulin resistance.

6.2. Diacylglycerol acyltransferase-1 in other organisms

DGAT-1 genes have been studied in other organisms. A polymorphism in the DGAT-1 promoter region is associated with a lower body mass index in Turkish women but not men [168]. In cows, the polymorphism K232A in DGAT-1 is strongly associated with a quantitative trait locus for decreased milk fat content [169,170]. In Drosophila, a DGAT that is 33% identical to human DGAT-1 and contains the conserved FYxDWWN motif, was identified as the mutant gene (*midway*) that causes premature apoptosis and degeneration of nurse cells [171].

6.3. Diacylglycerol acyltransferase-2

The existence of a second DGAT was suspected because DGAT-1$^{-/-}$ mice have normal plasma TAG levels, store TAG in fat cells, and maintain some DGAT activity in most tissues. When two novel DGAT isoforms without sequence homology to DGAT-1 were purified and cloned from the oleaginous fungus *Mortierella rammaniana* [172], the mouse and human homologs were sought. DGAT-2 was cloned by using the fungal DGAT-2 sequence to search the EST data base. The proteins from mouse and human are predicted to be 388 and 387 aa, respectively, and to have two predicted transmembrane domains [134]. DGAT-2 is said to have some similarities to Motif III in members of the glycerolipid acyltransferase family [57], though the correspondence is not impressive. In human tissues, two mRNA species (2.4 and 1.8 kb) are detected, and highest mRNA expression is observed in liver, mammary gland, adipose tissue, testis, and peripheral leukocytes, with lower expression in adrenal, thyroid, and brain. In mouse, highest expression is observed in heart, liver, and brown and white adipose tissue, with lower levels in many other tissues. Of particular interest is the presence of DGAT-2 in mouse, but not human small intestine. Similar to DGAT-1, DGAT-2 expressed in Sf9 insect cell membranes is dependent on *sn*-1,2-diacylglycerol and acyl-CoA as substrates. Specific activity is about twice as high with 18:1-CoA than with 16:0-, 18:2-, or 20:4-CoA. DGAT-2 mRNA increases 30-fold during 3T3-L1 adipocyte differentiation.

6.4. Diacylglycerol transacylase and wax ester synthase

sn-1,2(2,3)-Diacylglycerol transacylase was purified 550-fold from microsomal membranes of rat intestinal mucosa cells. The activity does not require an acyl-CoA, and lipase/esterase inhibitors partly inactivate the activity [148]. This enzyme has not been cloned.

Although TAG stores accumulate in several bacterial species (*Mycobacterium* [5], *Nocardia* [6], *Rhodococcus* [7], and *Streptomyces* [4]), the synthetic enzymes have not been identified. *Acinetobacter calcoaceticus* ADP1 accumulates both wax ester and TAG, and a bifunctional enzyme (WS/DGAT) was identified that exhibited both wax ester synthase and DGAT activities [149]. The predicted 458-aa protein, which is not related to other acyltransferases including the DGAT-1 or DGAT-2 families, contains a putative active-site motif (^{133}HXXXDG^{138}) resembling a conserved condensing domain found in enzymes that synthesize peptide antibiotics (NCBI Conserved Domain Data Base accession pfam00668) as well as similarity to the GPAT family Motifs I-IV (Table 2). Because the bacterial WS/DGAT contains all four motifs, its mechanism of catalysis may be similar to that of the GPAT family. The protein has a predicted transmembrane domain that lies towards the middle region of the protein. Expression of WS/DGAT in *E. coli* shows low DGAT activity with acyl-CoAs of 12–20 carbons; surprisingly, the highest activity was observed with 20:0-CoA, but the diacylglycerol species used as an acyl acceptor, *sn*-1,2-di16:0-glycerol, is perhaps not ideal for a DGAT assay [1]. A related sequence has not been identified in the human genome although wax synthesis occurs in mammals [173].

6.5. Stearoyl-CoA desaturase-1

Although not in the glycerol-3-P pathway of TAG synthesis, stearoyl-CoA desaturase-1 (SCD1) (EC1.14.19.1), which catalyzes the conversion of 18:0-CoA to 18:1-CoA (or 16:0-CoA to 16:1-CoA), plays an important, albeit as yet unclear, role in the synthesis of TAG. SCD-1 is expressed in most organs, and its activity decreases with starvation and insulin deficiency, and increases with diets enriched in saturated fatty acids [174]. Polyunsaturated fatty acids diminish SCD-1 mRNA and SREBP-1 increases it, consistent with an important role of SCD-1 in TAG synthesis under conditions in which lipogenesis is enhanced [174]. This role is observed in asebia mice, which have a natural mutation of SCD-1, and in SCD-1 knock out mice. Asebia homozygotes have low hepatic content of cholesterol esters and TAG compared with heterozygotes, as well as a plasma total cholesterol that is 50% higher than controls and a plasma TAG 60% lower than controls [175]. When asebia mice are fed a lipogenic diet (14% maltodextrin, 55% sucrose), SREBP-1, fatty acid synthase (FAS), mtGPAT expression, and liver TAG content increase in the heterozygote but not in the −/− asebia mice [176]. Surprisingly, supplementation of the lipogenic diet with triolein (50% total fat) for 2 days increases the percent of 18:1 in liver TAG from the asebia mice, but does not increase TAG content [176], suggesting that exogenously supplied 18:1 does not enter a correct pool or that there is an absence of a particular 18:1 enriched phospholipid species. When a targeted disruption is made of SCD-1 on the Sv129 background, the knockout mice, like the asebia mice, have reduced body fat, increased sensitivity to insulin and resistance to diet-induced obesity [177]. Energy expenditure and ketone production are increased, consistent with an increase in lipid oxidation as well as the decrease in TAG synthesis [177]. Because SCD-1 activity is increased 7-fold in *ob*/*ob* mice, asebia SCD1$^{-/-}$–*ob*/*ob* mice were studied [178]. The resulting double knockout mice weigh less than control *ob*/*ob* mice and have increased energy expenditure, suggesting that some of leptin's effects occur via down-regulation of SCD-1 activity [178]. How SCD-1 exerts these effects on weight and TAG synthesis remain unexplained.

Three additional mammalian stearoyl-CoA desaturases have been cloned but their contribution to TAG synthesis has not been investigated.

7. Topography of TAG synthesis and VLDL biogenesis

Before any of the glycerolipid synthetic enzymes were cloned, biochemical studies were used to determine that the active sites of the microsomal enzymes of glycerolipid synthesis, microsomal MGAT, GPAT, AGPAT, PAP, DGAT, acyl-CoA synthetase and the terminal enzymes of phospholipid synthesis all face the cytosol [179]. This conclusion was reached because (1) the activities are not latent when microsomes are permeabilized by detergent; (2) the activities are inactivated by proteases under conditions in which the microsomes remain impermeable to the proteases employed; (3) the long-chain acyl-CoA substrate of GPAT, AGPAT, MGAT, and DGAT cannot penetrate into the lumen of the microsomal vesicles [180,181]; and (4) other substrates of several of these enzymes (CMP, ATP, CDP-choline) cannot penetrate the lumen [179]. Data from inhibitor and palmitoyl-CoA-agarose studies suggested that mitochondrial GPAT also has an active site that faces the cytosol [182,183], and this topography was confirmed by studies with epitope-tagged recombinant protein [69]. Because the TAG synthesized in liver may be secreted as part of a VLDL particle or stored in cytosolic droplets, the question has arisen as to how TAG is moved from its site of synthesis on the cytoplasmic face of the ER into the ER lumen. Complicating this question are data suggesting that much of secreted VLDL–TAG originates from cytosolic TAG rather than from new synthesis from exogenous fatty acid [184]. Thus, functionally separate pools of TAG may exist. The question remains whether VLDL biogenesis "pulls" TAG out of the ER membrane (which can hold up to 3 mol% TAG [185]) or whether TAG destined for incorporation into VLDL must be synthesized on the lumenal surface. Although proteases inactivate DGAT in intact microsomes that are impermeable to the protease [181], new data on DGAT activity in the presence and absence of detergent have been interpreted to suggest that DGAT active sites are present on opposite sides of the ER membrane [186]. Additional studies by the same group report independent changes in "overt" and "latent" DGAT activities in neonatal liver, although no changes were observed in DGAT-1 and -2 mRNA [187], or in *ob*/*ob* mice [188]. These studies employed the substrate *sn*-1,2-di16:0-glycerol, a substrate that at 100 μM gives a DGAT specific activity of only about 6% of what is obtained using *sn*-1,2-di18:1glycerol [1].

It has been suggested that an ER-located carnitine palmitoyltransferase converts acyl-CoA to acyl-carnitines which are transported into the lumen to provide the substrate for a DGAT with an active site that faces the ER lumen [189]. Because CoA does not permeate microsomes, this study added CoA to the microsomal lumen via vesicle fusion; thus, a remaining problem is how CoA would normally be available for acyl-CoA resynthesis from acyl-carnitine. That a mechanism for providing ER acyl-CoA does exist can be inferred by the lumenal location of the ethanol acyltransferase [180]. Finally, new evidence has been reported for the existence of a system that transfers TAG across the membrane: the antibiotic verapamil blocks secretion of apoB-containing lipoprotein particles from HuH-7 human hepatoma cells, and blocks the transfer [^{14}C]TAG from cytosol to the lumen of isolated microsomes [190].

Topographical studies of DGAT-1 and DGAT-2 and identification of their active site residues should shed light on these questions as well as additional information on the putative TAG transport "machinery" and the role of ER carnitine palmitoyltransferase. It will also be important to investigate other specific proteins like microsomal triglyceride transfer protein that direct the flow of newly synthesized TAG into the lumen, together with other microsomal enzymes that might contribute to TAG synthesis or hydrolysis, like diacylglycerol transacylase and TAG hydrolase.

8. Regulation of triacylglycerol synthesis

The regulation of TAG formation and hydrolysis is likely to be complex, involving transcriptional and post-transcriptional controls that respond to specific hormones, to metabolites derived from individual meals and long-term diet exposures, to fasting and refeeding, and to exercise-mediated energy expenditure [71]. Regulation is likely, however, to differ greatly in different tissues. For example, TAG synthesis during milk biogenesis in breast epithelial cells is not regulated in the same manner as TAG synthesis in liver or adipocytes. In fact, breast milk retains its usual TAG content even during moderate deficits in dietary calories and protein [191] when TAG synthesis in liver and fat cells is down-regulated. In addition, regulation of TAG synthesis in liver probably varies physiologically throughout the life cycle as the primary energy source varies from carbohydrate prenatally, to very high-fat during the suckling period, and to a high carbohydrate diet after weaning. Important roles are played by SREBP-1c, PPARγ, and LXR, by their ligands, and by hormonal and nutritional regulators including insulin, carbohydrate, and fatty acids. It is not known whether the recently described carbohydrate-responsive element binding protein (ChREBP) [192] interacts with sites on the promoters of the genes that encode the TAG synthetic enzymes. For the most part, post-translational regulation is only inferred; few studies have directly addressed the cloned enzymes in the glycerolipid synthetic pathway.

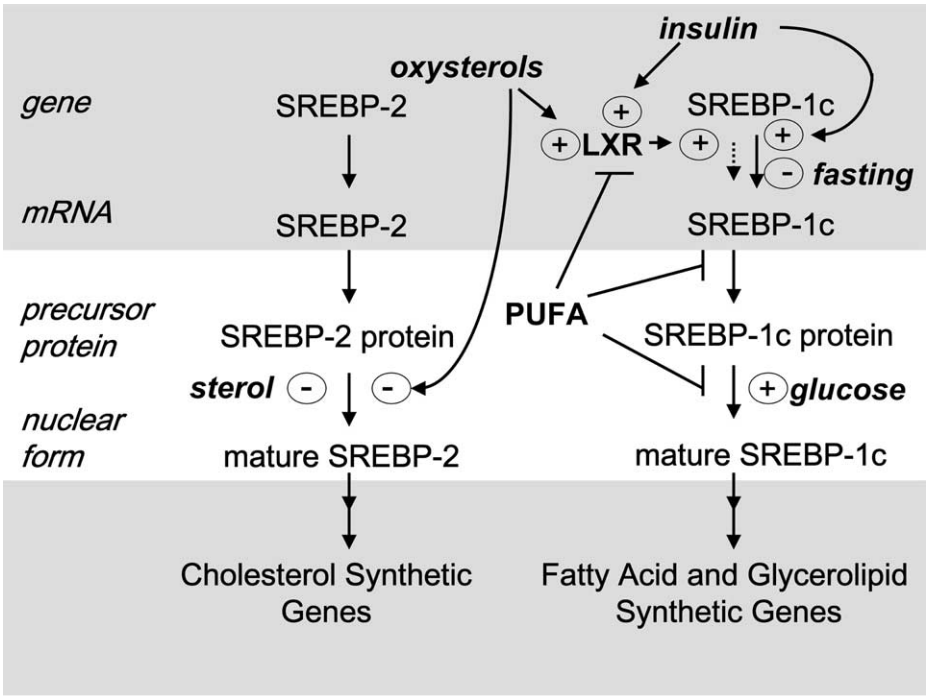

Fig. 3. Regulation of lipogenic and triacylglycerol genes by SREBP, LXR, polyunsaturated fatty acids, glucose, and insulin.

8.1. Sterol regulatory element-binding protein (SREBP)

Cholesterol metabolism and transport and fatty acid and glycerolipid biosynthesis are regulated by isoforms of the sterol regulatory element-binding protein (SREBP) [193,194]. The major SREBP isoforms in tissues are the independent gene products SREBP-1c and SREBP-2. SREBP-1c and SREBP-2 are the major forms in tissues, whereas SREBP-1a and SREBP-2 predominate in most cultured cell lines [194]. SREBP-1a, a variant of SREBP-1 produced via an alternate promoter, has an amino acid extension at the N-terminal transactivating domain, binds more strongly to SRE sites than SREPB-1c, and has a range of target genes in pathways of cholesterol, fatty acid and glycerolipid synthesis (Fig. 3) [195]. In contrast, SREBP-1c activates genes involved in fatty acid and TAG synthesis but not those in the cholesterol synthetic pathway, and SREBP-2 targeted genes are primarily those involved in cholesterol biosynthesis [193].

SREBPs bind to sterol regulatory elements (SREs) or to palindromic E box motifs in the promoter regions of target genes. These elements have been identified in the promoters of the lipogenic enzymes FAS [196,197], ACC [198], long-chain fatty acyl-CoA elongase [199,200] and SCD-1 [201]. Of the enzymes specific to phospholipid and TAG biosynthesis, SREs are present in the promoters of genes encoding mitochondrial GPAT [73] and the rate-controlling step in phosphatidylcholine synthesis, the CTP:phosphocholine cytidylyltransferase [200,202]. SREs have not been reported for other enzymes in the pathway of glycerolipid biosynthesis, although DGAT activity increases in rats after treatment with atorvastatin and simvastatin, drugs that indirectly up-regulate SREBP-2 [203].

Regulation by SREBP depends on the amount of membrane-bound SREBP precursor present in the endoplasmic reticulum and the rate of its cleavage to release the mature soluble protein, the N-terminal domain of SREBP (Fig. 3). Mature SREBP moves to the nucleus, forms a heterodimer with RXR, and binds to the SREs of target genes. Like SREBP-2, the amount of mature SREBP-1c depends on post-translational protease cleavage that releases the mature protein from its initial location in the endoplasmic reticulum [193]. Unlike SREBP-2, however, the amount of mature SREBP-1 peptide that reaches the nucleus is not primarily controlled by sterol-mediated protease cleavage. Instead, transcriptional regulation appears to play the major role [194]. The amount of cellular SREBP-1c is controlled by feed-forward regulation, liver X-activated receptors (LXRs), glucagon, and insulin. Although the promoter region of SREBP-1c contains a sterol-responsive region, this site may not be physiologically relevant because mouse liver SREBP-1 mRNA expression is not altered by a cholesterol-rich diet that would cause hepatic cholesterol to accumulate [204]. More critical is the SRE complex that responds positively to mature SREBP-1; this SRE complex may be responsible for the refeeding overshoot of the mRNA for SREBP-1c and its target lipogenic genes [204].

8.2. SREBP-1c regulation of TAG synthesis in liver and adipose tissue

It has long been observed that feeding a high carbohydrate diet upregulates hepatic lipogenesis and TAG synthesis, and, conversely, that fasting decreases these processes. These effects appear to be mediated in large part by the actions of insulin and glucagon on SREBP-1c, although the ChREBP could play a role as well. In rodents, feeding increases and fasting depresses hepatic SREBP-1c pro-

tein [196,205]. Insulin increases SREBP-1c and target gene mRNA in hepatocytes, and in streptozotocin-treated diabetic rats which do not produce insulin, SREBP-1c mRNA decreases [206]. When fasted animals are refed, the hepatic expression of lipogenic enzymes increases in normal, but not in SREBP-1c knockout mice [207]. In isolated hepatocytes, dominant-negative SREBP-1c inhibits the glucose-induced stimulation of pyruvate kinase, fatty acid synthase, and acetyl coenzyme A carboxylase mRNA, showing that SREBP-1c is required for the glucose effect on lipogenesis [208].

Liver responds to a 24 h fast by decreasing the mRNA of lipogenic enzymes like ACC, FAS, and SCD-1 and in the first regulatory step in TAG synthesis, the mtGPAT. If this 24 h fast is followed by 12 h of refeeding a high carbohydrate/ fat-free diet or by feeding the high carbohydrate/fat free diet for 2 weeks, these mRNAs normally increase markedly [209]. A knockout of the SREBP-1 gene, however, diminishes the fasting/refeeding changes with no alteration in the normal response of HMG-CoA reductase mRNA which is controlled by SREBP-2 [209]. In parametrial adipose tissue from SREBP-1 knockout mice, the changes in mRNA expression are attenuated but not as severely as in liver [209]. These data suggest that SREBP-1 is not needed for basal expression of lipogenic genes, but, instead, is required for changes induced by diet. Further, SREBP-1 is required for both short and long-term responses to diet. Conversely, when SREBP-1c is over-expressed, FA synthesis and target genes like fatty acid synthase increase 4-fold and mtGPAT increases 10-fold [195,210,211].

The transcriptional activator Adipocyte Determination and Differentiation factor (ADD1) is required for differentiation into mature fat cells [212]. ADD1 is identical to SREBP-1c [213,214]. A dominant-negative form of ADD1 sharply represses adipocyte differentiation [215]. Although SREBP-1c in rodents responds to feeding and fasting [196,205], in humans, insulin and carbohydrate do not increase adipose SREBP-1c mRNA and little de novo lipogenesis occurs [216]. Leptin reduces nuclear SREBP-1c mRNA in liver and adipose tissue [217,218].

8.3. Liver X receptors (LXR)

LXRα and β, are nuclear receptors that are activated by oxysterols and which decrease excess free cholesterol in cells by activating genes that control the rate of bile acid synthesis and cholesterol efflux. The SREBP-1c promoter contains an LXR binding site [219], and mice lacking LXRα and β express less SREBP-1c and its target liver genes [220]. Thus, when the concentration of cellular sterol increases, LXR activates SREBP-1 which increases the synthesis of oleate, possibly for use in the synthesis of cholesterol esters. Dietary fish oil, which suppresses plasma TAG in VLDL and chylomicra [221], appears to achieve its effect, in part, through the ability of PUFA to competitively inhibit the activation of LXR [222]. Lack of LXR activation causes a decrease in SREBP-1c transcription, thereby reducing lipogenesis and TAG synthesis [223]. Additionally, PUFA increase the rate of SREBP-1 degradation [224]. Thus, PUFAs lower SREBP-1c by both decreasing its rate of synthesis and increasing its rate of degradation.

8.4. Regulation by PPARγ and PPARα

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear transcription factor whose ligands include 15-deoxy-$\Delta^{12,14}$prostaglandin, fatty acids and the glitazone drugs [225]. As a part of its general effect in promoting the differentiation of adipocytes, PPARγ markedly upregulates the expression of most of the enzymes of lipogenesis and of TAG synthesis [225,226]. Although

not identified in the published gene arrays, the 3- and 70-fold increases in the specific activities of PAP-1 and of microsomal GPAT, respectively, after adipocyte differentiation [30] strongly suggest that these enzymes might be encoded by one of the unidentified ESTs of groups C and D in the 3T3-L1 gene expression study [226]. In addition to up-regulation of glycerolipid enzymes with adipocyte differentiation, further increases in mRNA can occur with PPARγ activation. When Zucker diabetic fatty rats are given a selective PPARγ agonist for 7 days, the mRNA of lipogenic enzymes and mtGPAT increase in white and brown adipose tissue [227]. PPARγ expression in liver is normally about 10–30% of that in adipose tissue [228], but its expression increases with hepatic steatosis related to PPARα deficiency [229], as well as murine models of obesity and type 2 diabetes mellitus [230]. Steatosis diminishes in patients with non-alcoholic steatohepatitis who are treated with a PPARγ ligand [231], but PPARγ1 overexpression in liver produces steatosis and increases in lipogenic genes, including DGAT-1, and genes normally expressed in adipocytes like aP2/adipose fatty acid binding protein and adiponectin [232]. These studies suggest that the steatosis occurs via PPARγ-mediated stimulation of lipogenesis. This idea is supported by studies of a liver-specific disruption of PPARγ on an *ob*/*ob* background [233]. The deficiency of PPARγ in these leptin-deficient mice resulted in a 75% decrease in liver TAG content and decreases in the mRNA for the lipogenic enzymes, fatty acid synthase, acetyl-CoA carboxylase, SCD-1, and mtGPAT, among others, but it is not known whether these effects are direct.

PPARα is a transcription factor that is also activated by fibrates and by fatty acids, particularly in fasted animals [234]. Clinical use of fibrates decreases serum TAG, but it is not known whether this effect is due to a direct effect on the enzymes of TAG synthesis. Studies of PPARα null mice show hepatic steatosis and diminished mRNAs for proteins involved in fatty acid oxidation, and it has been assumed that the increased hepatic TAG synthesis is a secondary effect of the diminished capacity for oxidation [235]. Changes in mRNA of enzymes in the TAG synthetic pathway have not been reported. Older studies suggest that several of the enzymes of glycerolipid biosynthesis can be regulated by PPARα. For example, the PPARα agonists clofibrate, gemfibrozol, and Wy-14,643 increases mouse liver microsomal GPAT activity 2.8-fold [236], and PPARα activators up-regulate liver acyl-CoA synthetase-1 mRNA [237]. Further, exposure to cold and sympathetic activity increase mtGPAT, AGPAT and PAP-1 specific activities 3-fold in brown adipose tissue [238]. These observations should be re-examined using molecular tools to directly determine the response of the mRNAs to PPARα and the presence of PPREs in the promoters of these genes.

8.5. Regulation by polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) decrease the expression of mRNAs for the lipogenic enzymes regulated by SREBP-1c, including acetyl-CoA carboxylase, fatty acid synthase, SCD-1, ATP citrate lyase, pyruvate kinase, and malic enzyme, as well as that of mtGPAT [239,240]. These changes are likely to be responsible for the beneficial effects of fish oil capsules on the markedly elevated TAG that occurs in human lipoprotein lipase deficiency and hypertriacylglycerolemia [241,242]. The mechanism by which PUFA decreases TAG synthesis is just beginning to emerge. PUFA decreases the amount of SREBP-1 mature protein [243]. Fish oil feeding decreases SREBP-1a and-1c mRNA, inhibits proteolysis of the SREBPs [244,245], and also increases the rate of their transcript decay [224]. A portion of these effects are mediated by LXR. Fatty acids block the ligand-dependent activation of LXR which is required for SREBP transcription (Fig. 3).

The saturated fatty acid 18:0 has no effect, but other fatty acids are better blockers, in the order: 18:1 < 18:2 < 18:3 = 20:4 at 10–500 μM (For 20:4 and 18:3, 1/2 max inhibition of LXR occurred at < 10 μM). These effects appear to explain the mechanism by which unsaturated fatty acids decrease liver synthesis and secretion of fatty acid and TAG [222]. It has not been determined whether the fatty acids themselves or their acyl-CoA metabolites are the actual LXR antagonists.

8.6. Regulation and function in heart, and adipose tissue

TAG as a source of energy must be packaged into lipoproteins and delivered to different tissues from its synthetic sites of origin in intestinal mucosa cells and liver. Circulating chylomicon–TAG and VLDL–TAG are an important energy source for the heart, since cardiac muscle derives 60–70% of its energy from fatty acids [246]. Endogenous myocardial TAG is a critical fuel storage depot since it provides 10–50% of the energy requirements of the heart [247]. Fasting, obesity, and diabetes are conditions in which serum fatty acids and myocardial fatty acid uptake are elevated [248–251]. In these pathophysiological states, TAG synthesis is upregulated and myocardial TAG content increases 2–6-fold [252,253]. The heart also secretes apoB100 containing lipoproteins which may provide a mechanism to unload excess TAG [254–256].

The regulation of TAG synthesis probably differs in white and brown adipocytes. For example, fat stores in white adipose tissue increase during times of caloric excess and are depleted during caloric deficiency. In contrast, although the stores of TAG in brown adipocytes are depleted in hibernating animals that fast throughout the winter, the fact that the white adipose tissue TAG declines dramatically during the winter fast implies that, in addition to being oxidized by shivering skeletal muscle, some fatty acid released from white adipose tissue enters brown adipose tissue for resynthesis to TAG, so that brown adipose tissue stores will be able to support the next rewarming period.

But there are additional sources of TAG synthesis that have been poorly studied. The DAG signal can be attenuated by conversion to TAG [120]. It is not known whether this conversion is catalyzed by DGAT-1, DGAT-2, DAG transacylase or PC transacylase. It is also possible that LPA, which can enter cells and interact with PPARγ to alter gene expression [257], may be able to enter the synthetic pathway and become converted to TAG. These routes have not been investigated.

8.7. Regulation of special processes

8.7.1. Triacylglycerol in milk

TAG is a major source of energy in the breast milk delivered to growing infants. The content of milk TAG varies from a low of 2 g/l in the rhinoceros to a high of 500 g/l in seals whose milk contains no lactose, the second major caloric source in milk [258]. The structure of milk TAG differs from that of TAG synthesized in other organs because differentiated mammary epithelial cells express a specialized thioesterase II that cleaves fatty acids prematurely at 12 or14 carbons [258]. These medium-chain fatty acids are esterified at the *sn*-3 position of milk TAG, and milk from women eating a low fat diet contains high amounts of medium-chain fatty acids [259,260]. In contrast, when a high fat diet is consumed, esterification favors fatty acids derived from the diet, so that milk TAG contains relatively low quantities of medium-chain fatty acids. A striking

example was observed in a woman who could not use dietary fatty acids because her deficiency in lipoprotein lipase impaired hydrolysis of VLDL and chylomicra TAG. Her milk contained 39.5% C12:0 and C14:0 compared to the usual 10.7% in milk from control women eating a similar diet [261].

The production of TAG in milk depends on hormone-induced up-regulation of the enzymes of TAG synthesis, but no studies have investigated the now-cloned enzymes, except for DGAT-1 whose absence completely blocks milk production [161]. Constitutively activated AKT in mammary gland results in a milk fat content that is 65–70% of energy instead of 25–30% as in wild type mice [262]. Glucose uptake and incorporation into TAG is enhanced but it is not known whether the enzymes of TAG synthesis are up-regulated.

8.7.2. Neonatal liver

Fetal animals live on a high glucose diet that contains relatively little fatty acid; at birth, the primary energy source switches abruptly to TAG. Concomitantly, in postnatal rats, the activities of the enzymes of β-oxidation and ketone body formation increase 10-fold [263–265]. Between prenatal day 4 and postnatal day 8, specific activities of enzymes of glycerolipid synthesis in rat liver increase dramatically: microsomal GPAT activity increases 74-fold, and large increases are observed for acyl-CoA synthetase, MGAT, AGPAT, mtGPAT, and DGAT [35,131]. In the second group, every enzyme activity is comprised of two or more isoforms; thus, specific mRNA changes should be now be examined. In suckling rat pups at day 15, nuclear SREBP-1 protein levels are low, despite amounts of mRNA that are as high as observed in adult animals. The nuclear levels increase after weaning, as does the representative lipogenic target gene for fatty acid synthase. Thus, it is surprising that the mRNA for mtGPAT, the representative TAG synthesis target gene, is 2 to 3-fold higher on days 15–18 than it is on day 30 after weaning [266]. The discrepancy between fatty acid synthase and mtGPAT suggests that SREBP-1c may not the major regulator of GPAT in the neonatal period and that other transcription factors may be more important. In fact mRNA expression peaks during the mid suckling period. This pattern of expression suggests regulation by PPARα because of the similarity to the expression of mitochondrial HMG-CoA synthase, the rate-limiting step in ketogenesis [266,267].

9. Lipid associated toxicity

The accumulation of TAG in liver and non-adipocyte peripheral tissues, particularly skeletal muscle, is highly associated with insulin resistance and apoptosis [268]. At present it is uncertain as to whether TAG is a culprit, an innocent bystander, or a benefactor. Many of the studies are observational, but a few directly attempt to increase or decrease TAG production or alter the amounts of the putative direct lipid mediators. TAG accumulation in non-adipose tissue in obesity and diabetes is regarded at best, as a marker of lipid toxicity and, at worst, as a source of toxic lipid mediators. For example, the islets of leptin-unresponsive *fa/fa* Zucker diabetic fatty rats contain up to 100-fold more TAG than do normal rat islets and undergo apoptosis that can be blocked by triacsin C, an inhibitor of some acyl-CoA synthetase isoforms [269] and, thus, an inhibitor of TAG synthesis [270,271]. The liver and pancreatic islets of *fa/fa* rats also express more hepatic SREBP-1c mRNA as well as mRNA of

the lipogenic enzymes fatty acid synthase, acetyl-CoA carboxylase, and mtGPAT, [272]. When the *fa*/*fa* rats are given troglitazone, the expression of these enzymes decreases and diabetes is prevented. In normal rats with diet-induced obesity, hyperleptinemia prevents SREBP-1 over-expression and diminishes the lipogenic enzymes [272]. When pancreatic islets from the diabetic *fa*/*fa* rats are cultured with 1 mM fatty acid (18:1/16:0; 2/1), an increase in ceramide level is followed by measures of apoptosis; both are blocked by fumonisin B, an inhibitor of ceramide synthesis, suggesting that the fatty acid causes apoptosis via a ceramide-mediated pathway [273]. The *fa*/*fa* rats also accumulate TAG and ceramide in their hearts and exhibit contractile abnormalities that are ameliorated by troglitazone therapy [252]. Both exogenous fatty acid and TAG may be sources of diacylglycerol and acyl-CoAs that can activate protein kinase C and decrease insulin-induced, IRS-1-mediated PI3-kinase activity in skeletal muscle [274]. Increases in diacylglycerol mass in human skeletal muscle are also associated with decreases in IκB-α, which is up-regulated in oxidative stress [275]. Taken as a whole, these studies implicate TAG as a either a marker or source of toxic lipid products like ceramide and diacylglycerol that promote apoptosis.

On the other hand, arguing for a role of TAG as a benefactor in preventing apoptosis, the apoptosis induced by 20:4 in 293 cells can be prevented by over-expression of acyl-CoA synthetase-4 which preferentially activates 20:4 fatty acid. In cells that over-express acyl-CoA synthetase-4, ceramide synthesis decreases, presumably because more fatty acid is incorporated into glycerolipids, although this incorporation was not actually measured [276]. In this model, triacsin C, which inhibits acyl-CoA synthetase-4, increases, rather than decreases apoptosis. In another study, the cytotoxicity of oleate and palmitate in isolated normal rat islet β-cells is associated with less TAG accumulation, and the authors speculate that the fatty acids themselves are toxic and that formation of cytosolic TAG from exogenous fatty acid protects against fatty acid-induced apoptosis [277]; further, when added directly to cultured β-cells, troglitazone actually increases apoptosis, suggesting that the protective effect of this agent against fatty acid toxicity might be indirect [278]. Similarly, 16:0-induced apoptosis of CHO and mouse embryonic fibroblast cells is achieved independent of ceramide production; and adding 18:1 to the media increases 16:0 incorporation into TAG and protects against apoptosis [279,280]. The latter studies suggest the possibility that poorly metabolized fatty acids like 16:0 might decrease cellular ATP levels and/or trap CoA as acyl-CoA. Increasing the conversion of fatty acid to TAG may regenerate free CoA and allow critical metabolic processes like the TCA cycle and oxidative phosphorylation to proceed. Arguing against the beneficial effect of enhanced TAG synthesis, however, DGAT-1 over-expression in rat pancreatic islets increases the incorporation of 16:0 into TAG 2-fold and causes the islet insulin secretion to respond poorly to glucose independent of exposure to 16:0, another mechanism of impairing insulin secretion [281]. The authors concluded that TAG content itself mediated part of the toxic effect of fatty acid.

10. Lipid intermediates as signals

Although it has not escaped notice that three of the intermediates in the glycerol-3-phosphate synthetic pathway are signaling molecules, little evidence exists that the lysophosphatidic acid, phosphatidic acid or DAG produced as glycerolipid intermediates are part of a signaling path-

way. As signals, lysophosphatidic acid and phosphatidic acid are released externally from plasma membrane phospholipids and become ligands for cell surface receptors that initiate G-protein-coupled intracellular pathways in neighboring cells [282,283]. Similarly DAG is released via phospholipase C from plasma membrane phospholipids to activate protein kinase C. Whereas a major function of phospholipid is to form the building blocks of biological membranes, a small population of lipids has been implicated as signaling molecules acting either as intracellular second messengers or as extracellular agonists that modulate cell function [284,285]. Lysophosphatidic acid in serum can be produced from platelet and serum phospholipids via a number of different phospholipase pathways [286]. Because lysophosphatidic acid is also a ligand for PPARγ [257], questions now arise about whether the lysophosphatidic acid that is produced intracellularly de novo via the glycerol-3-phosphate pathway can alter gene expression.

11. Perspectives

Although much has been learned in the 3 years since our last review of this topic [71], much remains to be investigated. At least two genes, those for the microsomal GPAT and PAP-1 have still not been identified, and the putative AGPATs-3,-4,-5, and-6 have not been cloned or studied. The subcellular locations of each of the enzymes that have more than a single isoform have not been confirmed, and, except for mtGPAT, topographical studies of these intrinsic membrane proteins have yet to be performed. With the growing ability to crystallize membrane proteins, it will be important to determine the active site structures of each of the enzymes and learn how they interact with their substrates in the plane of the membrane. Do each of the AGPAT, DGAT and MGAT family members have different non-overlapping functions? Are their substrates and products segregated in separate pools or do they form subcellular protein complexes that channel intermediates into specific pathways? Do each of the GPAT, AGPAT, DGAT, and MGAT isoforms form heteromers?

Since TAG plays decidedly different roles (compare liver, white and brown adipose, adrenal, intestinal mucosa, lactating mammary gland), the enzymes of TAG synthesis should be regulated independently in different tissues both transcriptionally and post-transcriptionally. How does this occur? Although numerous studies suggest that several of the enzymes of TAG synthesis are acutely regulated by cAMP-dependent protein kinase and other hormonally regulated kinases [71], and that recombinant AMP-activated kinase decreases mtGPAT activity [78], no one has directly studied acute regulation of the cloned GPAT, AGPAT, MGAT, or DGAT isoforms.

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