**The DGA1 Gene Determines a Second Triglyceride Synthetic Pathway in Yeast**

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Diacylglycerol esterification provides an excellent target for the pharmacological reduction of triglyceride accumulation in several human disease states. We have used *Saccharomyces cerevisiae* as a model system to study this critical component of triglyceride synthesis. Recent studies of an oleaginous fungus, *Mortierella ramanniana*, identified a new family of enzymes with *in vitro* acyl-CoA:diacylglycerol acyltransferase activity. We show here that *DGA1*, the sole member of this gene family in yeast, has a physiological role in triglyceride synthesis. Metabolic labeling of *DGA1* deletion strains with triglyceride precursors detected significant reductions in triglyceride synthesis. Triglyceride synthesis was virtually abolished in four different growth conditions when *DGA1* was deleted in concert with *LRO1*, an enzyme that esterifies diacylglycerol from a phospholipid acyl donor. The relative contributions of the two enzymes depended on growth conditions. The residual synthesis was lost when *ARE2*, encoding an acyl-CoA:sterol acyltransferase, was deleted. *In vitro* microsomal assays verified that *DGA1* and *ARE2* mediate acyl-CoA:diacylglycerol acyltransferase reactions. Three enzymes can thus account for diacylglycerol esterification in yeast. Yeast strains deficient in both diacylglycerol and sterol esterification showed only a slight growth defect indicating that neutral lipid synthesis is dispensable under common laboratory conditions.

A common element to diabetes, atherosclerosis and obesity, is the subcellular and extracellular accumulation of neutral lipids (1–3). The synthesis of these neutral lipids (triglyceride and sterol ester) results in both storage and detoxification of the alcohol and acyl substrates to these reactions. The reactions thus represent a pivotal component of lipid homeostasis in all eukaryotes and of the pathophysiology of some of the most prevalent human disease syndromes of the western world (reviewed in Refs. 4–6).

Significant progress has been made recently toward the complete identification of the enzymes that mediate the intracellular synthesis of cholesteryl esters and triglyceride (TG). In mammals, these reactions are catalyzed, in part, by the three members of the *O*-acyltransferase gene family (5, 7–12). The ACAT1 and ACAT2 genes encode a ubiquitous and a tissue-specific acyl-CoA:cholesterol *O*-acyltransferase (ACAT), respectively (9, 10, 12), while the DGAT1 gene encodes an acylcoenzyme A (CoA) diacylglycerol *O*-acyltransferase (DGAT (9, 11, 13, 14)). Although induced mutant mice for DGAT1 exhibit reduced body fat and are resistant to diet-induced obesity, these animals have normal serum TG levels, indicating that DGAT1-independent mechanism(s) for TG synthesis must also exist (15, 16). Such a mechanism may be mediated, at least in part, by DGAT2, a human protein recently identified based on sequence similarity to two acyl-CoA:diacylglycerol acyltransferases purified from the oleaginous fungus *Mortierella ramanniana* (16–18). The two *M. ramanniana* enzymes are 53% identical and show no sequence similarity to the *O*-acyltransferase gene family. Together with their orthologs in *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae* (*DGA1*), and humans (DGAT2), these enzymes comprise the DGAT2 gene family. The heterologous over expression of these proteins, including *DGA1*, in insect cells conferred diacylglycerol acyltransferase activity *in vitro* (16–18); however, this experiment does not address the *in vivo* contribution of the DGAT2 family to TG synthesis.

In the yeast *S. cerevisiae*, the two members of the *O*-acyltransferase gene family are the ACAT-related enzymes, *ARE1* and *ARE2* (19–22). While *Are1p* and *Are2p* mediate the esterification of ergosterol and its precursors (23), deletion of both genes did not detectably reduce *3*Holeate incorporation into TG (19, 21). Rather, a novel reaction for TG synthesis mediates the esterification of diacylglycerol (DG) using phosphatidylcholine or phosphatidylethanolamine as the acyl donor. Lro1p, the yeast protein that catalyzes this phospholipid:diacylglycerol acyltransferase (PDAT) reaction, bears striking sequence similarity to mammalian lecithin cholesterol acyltransferase (21, 22). This reaction has also been identified in plant cells as a likely contributor to TG synthesis (22). Deletion of *LRO1* results in a significant reduction of TG synthesis. The TG biosynthesis that persists in the absence of *LRO1* is predominately acyl-CoA-dependent and did not diminish in the absence of *ARE1* and *ARE2* (21). *DGA1*, the yeast member of the DGAT2 gene family, is therefore a good candidate for mediat-
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LRO1-independent TG synthesis in yeast.

The studies described here demonstrate that DGA1 has a genuine physiological role in TG synthesis and that its relative contribution varies with growth conditions. In all conditions assayed, the combined deletion of DGA1 and LRO1 resulted in virtual loss (≥97% reduction) of TG synthesis. The residual TG synthesis was completely lost when ARE2 was deleted in conjunction with DGA1 and LRO1. Thus, the enzymes that esterify DG to form triglyceride in S. cerevisiae are now identified in full.

EXPERIMENTAL PROCEDURES

General—Molecular biology and yeast genetic procedures were performed according to conventional protocols (24). Yeast extract, yeast nitrogen base, bacto-peptone, and bacto-agar were from Difco. N-Dextrose, and n-raffinose were from Sigma. Complete (YPD yeast extract, peptone (YP), plus 2% dextrose), synthetic complete, YPRT0 (YP + 2% (w/v) raffinose, 0.2% (w/v) Tween 40, 0.1% (w/v) olate), and sporulation media (0.3% (w/v) potassium acetate, 0.02% (w/v) raffinose) were prepared as described (24, 25). Fatty acyl-CoAs, triolein, and 1,2-dioleoyl-sn-glycerol were from Sigma. [2-3H]Glycerol, [9,10-3H]N(oleic acid, [3H]Triolein, and [1-14C]Cleoyl-CoA were from PerkinElmer Life Sciences or Amersham Biosciences. Sequence searches were performed using the tBLASTn program (26), and comparisons performed using the GAP and CLUSTALW program (27, 28). Neutral lipid vital staining of yeast was performed using Nile Red as described previously (29).

Yeast Strains—Transformation of yeast was performed with lithium acetate followed by prototrophic selection (29). Yeast strains with single or multiple deletions (SCY060, SCY061, SCY059, SCY1397) in the ARE or LRO1 genes have been described previously (19, 21). Deletion mutant strains for YOR245C (DGA1) were generated by homologous recombination in a W303-1B haploid (MATa ade2-1, can1-1, trpl-1, ura3-1, his3-11, 15, leu2-3, 112, 244 a, 1, 2a) genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA. The 1.8-kb PCR product was generated using the oligos: TATTCCTGTAAG, and W303-1B genomic DNA.

Yeast Expression Plasmid Construction—A PCR product including the yeast DGA1 ORF and 3′-flanking sequence and 98 bp of 3′-flanking sequence was generated using the primers 245KOF, TATATATATACAAAGGAAACGCAGAGGCATACAGTTTG (245KOf, CCAATGAATT-245KO), and TACATATACATAAGGAAACGCAGAGGCATACAGTTTG (245KOf, CCAATGAATT-245KO) were confirmed by PCR and subsequently crossed with SCY1397 (Kluyveromyces lactis URA3 ura3-1, his3-11, 15, leu2-3, 112, DGA1 or multiple deletions (SCY060, SCY061, SCY059, SCY1397) in the peroxisome. 

Neutral lipid vital staining of yeast was performed using Nile Red as described (29). Yeast cells were grown in synthetic complete, YPR (YPD) or YPD cultures grown to stationary phase (OD660, 3.5 ± 0.4), washed twice in sterile dH2O, resuspended in sporulation media with [3H]glycerol (10 μCi/ml) and [3H]oleate (0.05 μCi/ml), and grown for 24 h at 30°C. Cells were harvested, washed, and the lipids extracted and separated by thin layer chromatography (TLC) as described previously (21). Assays were performed on a minimum of two independent strains of each genotype on two different days. To normalize for variability in uptake and extraction efficiency, the data are normalized (as %) to total lipid per lane of the TLC plate. Statistical analysis was performed using t-tests.

In Vitro TG Synthesis Assays—Microsomes were prepared as described previously (21, 32). All assays were performed in a final volume of 200 μl for 10 min at 23°C following a 15-min preincubation as described (33). The assay contained 175 mM Tris, pH 7.8, bovine serum albumin (0.2 mg/ml), 160 μM DG (in ethanol), 8 mM MgCl2, 50 μM [1-14C]oleoyl-CoA (20,000 dpm/nmol), and 80 μg of microsomal protein. To investigate fatty acyl-CoA specificity, microsomes were incubated with 1-steinoyl-2-[1-14C]arachidonyl-sn-glycerol (Amersham Biosciences, 15,000 dpm/nmol) and varying fatty acyl-CoAs (50 μM). Reactions were stopped by the addition of chloroform/methanol (2:1). 15 μg of [3H]triolien was added as an internal standard/carrier, and the lipids separated by TLC in hexane/diethyl ether/acetic acid (180:20:1). The background was determined as the activity observed when the radiolabel was added after the chloroform/methanol.

RESULTS AND DISCUSSION

DGA1, the Yeast Ortholog of the DGAT2 Gene Family—The sole representative of the DGAT2 gene family in S. cerevisiae, DGA1 (YOR245C), is predicted to encode a 418-amino acid polypeptide with ~36% identity to the MrDGAT2A and MrDGAT2B proteins from M. ramannii. Yeast Dga1p, in common with the other members of the DGAT2 family, is a predicted transmembrane protein, lacks a classical signal peptide and retains motifs and residues implicated at the active sites of bacterial glycerol-3-phosphate acyltransferases (34). The heterologous overexpression of S. cerevisiae, DGA1 has been used to infer this gene product to possess DG esterification activity (18). To determine the physiological role of Dga1p in triglyceride synthesis in yeast, we generated a DGA1 deletion mutant (dga1Δ) by homologous recombination. The dga1Δ strain was assessed for changes in TG production by metabolic labeling of exponentially dividing cells with [3H]oleic acid. The contribution of DGA1 under these conditions was significant but minor, with a 25% reduction in TG synthesis upon deletion of the gene (Fig. 1A).

DGA1 and LRO1 Account for the Majority of TG Synthesis in Yeast—Previous assays of yeast strains lacking the PDAT reaction encoded by LRO1 showed an ~75% reduction in TG synthesis in logarithmically growing yeast (Fig. 1A, Ref. 21). To determine the combined roles of DGA1 and LRO1 in TG synthesis, we generated compound lro1Δ dga1Δ deletion strains and assessed [3H]oleic acid incorporation into TG. During logarithmic growth, all but ~2% of normal TG synthesis was lost due to these mutations (Fig. 1A). Concomitantly, there was a statistically significant accumulation of the diacylglycerol and phospholipid substrates for these reactions in lro1Δ dga1Δ deletion strains (Fig. 1B).

As yeast cells pass through the diauxic shift or transition from logarithmic growth into the stationary phase of growth, their cellular metabolism is altered in response to the changing nutrient environment. This alteration includes the elevation of TG synthesis (35, 36). We therefore tested the contribution of DGA1 and LRO1 to TG synthesis in late stationary phase (Fig. 1A). Deletion of DGA1 caused an ~50% reduction in TG synthesis whereas deletion of LRO1 did not significantly effect TG synthesis. Thus, Dga1p can mediate essentially all cellular TG synthesis in stationary phase, in contrast to the situation in exponential phase where Lro1p predominates. This may reflect the marked transcriptional up-regulation of DGA1 relative to LRO1 that is observed during the diauxic shift and stationary growth (37). Alternatively, in stationary phase, the amount of substrate may be limiting such that a reduction in Dga1p or Lro1p levels has less of an effect on TG synthesis. Again, when both genes were deleted in concert, a major reduction (97%) in synthesis of TG together with an accumulation of DG and phospholipid resulted.

To verify that deletion of DGA1 was the genuine cause of the TG synthetic defect, a DGA1 expression plasmid (pRS413-DGA1) comprising the DGA1 ORF and endogenous promoter was created in pRS413. [3H]Oleic acid pulse-labeling of dga1Δ
or lro1Δ dga1Δ deletion strains transformed with either pRS413 or pRS413-DGA1 showed that plasmid-derived expression of DGA1 rescued the TG synthetic deficit (Fig. 2).

**DGA1 and LRO1 Differentially Contribute to TG Synthesis in Oleate-rich or Nitrogen-poor Media**—TG biosynthesis is also markedly elevated when yeast cells are grown in the presence of oleate (38) or when diploid cells are induced to sporulate in the absence of nitrogen (39). Therefore, we tested the incorporation of [3H]glycerol into TG following growth with 0.1% (w/v) oleate or in sporulation media. Deletion of LRO1 or DGA1 individually had no effect on TG accumulation in oleate-containing media (Fig. 3A). Deletion of LRO1 also had no effect in sporulation media while deletion of DGA1 resulted in a 40% reduction (Fig. 3A). Metabolic labeling experiments with [14C]oleic acid gave similar results (data not shown). The non-additive, or in the case of oleate media, non-existent, reductions in TG synthesis in the individual deletion strains may again reflect regulation or non-limiting amounts of either enzyme. Deletion of both genes resulted in the virtual loss of TG synthesis in both media, suggesting the absence of other genes that contribute significantly to TG synthesis under these growth conditions (Fig. 3A). As observed in the previous growth conditions, the deficits in TG synthesis were associated with a striking accumulation of DG (Fig. 3B). This observation confirms DG as a critical metabolic branch point in the Kennedy pathway for glyceride and glycerophosphatide synthesis (40), whereby either TG can be synthesized for storage or phospholipids synthesized for membrane formation, depending on cellular requirements. Accordingly, the accumulation of both DG and phospholipid is much less pronounced when cells were exponentially dividing (Fig. 2B), presumably because the pathway is biased toward new membrane formation.

**Are2p Is Responsible for Residual TG Synthesis in the Absence of DGA1 and LRO1**—In all of the growth conditions examined, there persists a very low level of TG synthesis that is independent of DGA1 and LRO1. Given the role of the mammalian O-acyltransferase, DGAT1 in this process, we questioned whether the yeast O-acyltransferase gene family might be responsible for residual TG synthesis. Moreover, it has been suggested that the Are1p enzyme has this activity (41). To address this possibility, we generated dga1Δ lro1Δ are1Δ and dga1Δ lro1Δ are2Δ strains. These quadruple deletion mutants incorporated no [3H]oleic acid into TG during stationary phase (Fig. 4); TG dpm were equal to the background of the scintillation counter. Subsequent analysis of dga1Δ lro1Δ are1Δ and dga1Δ lro1Δ are2Δ

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**Fig. 1.** Dga1p and Lro1p are the major determinants of triglyceride biosynthesis in yeast. Normal (N), single or double deletion mutants of DGA1, and LRO1 were grown to logarithmic or stationary phases and pulse-labeled with [3H]oleic acid as described under “Experimental Procedures.” The data are presented as the percent (of total extractable lipids) incorporation into triglyceride (Fig. 1A), or diacylglycerol and phospholipid (Fig. 1B) with standard deviations. Lipid synthesis in an lro1Δ strain during logarithmic phase from reference (21) is shown for comparison purposes. The total dpm per TLC lane averaged 650,000 (logarithmic) and 120,000 (stationary). Asterisks denote statistically significant (p < 0.01) differences compared with normal strains.

**Fig. 2.** Vector-derived DGA1 expression confers triglyceride synthesis to deletion mutants. Normal, dga1Δ, or dga1Δ lro1Δ strains were transformed with either pRS413 or pRS413-DGA1 as indicated. These strains were transformed with [3H]oleic acid and assayed for triglyceride incorporation as described under “Experimental Procedures.” Asterisks denote statistically significant (p < 0.01) differences compared with vector transformed strains of the same genotype.
strains showed that Are2p, and not Are1p, is responsible for the residual TG synthesis. As anticipated, the triple *dga1Δ lro1Δ are2Δ* and quadruple *dga1Δ lro1Δ are1Δ are2Δ* mutants display significant reductions in steryl ester synthesis in the same metabolic labeling experiment (not shown).

Normal yeast strains grown to stationary phase exhibit a marked accumulation of cytoplasmic neutral lipid droplets that can be envisaged by staining with the vital stain Nile Red, followed by fluorescence microscopy (Fig. 5). By contrast, the same analysis of a *dga1Δ lro1Δ* mutant revealed a significant reduction in the number and intensity of lipid droplets (Fig. 5). Furthermore, the quadruple mutant displayed a complete absence of neutral lipid containing droplets (Fig. 5). Therefore, the dramatic effects of mutations in the *DGA1*, *LRO1*, and *ARE2* genes on neutral lipid accumulation are observed with experiments using two independent assays, metabolic labeling and Nile Red staining.

**DGA1 and ARE2 Use Acyl-CoA As a Substrate in the Synthesis of TG**—To assess the direct role of these enzymes in a classical DGAT reaction, microsomes were isolated from normal, single deletion, and compound deletion strains and incubated with DG and radiolabeled oleoyl-CoA (Table I). Even though *LRO1* does not use acyl-CoA as a substrate, yeast microsomes can generate DG by the hydrolysis of phosphatidate derived from glycerol-3-phosphate and acyl-CoA (42, 43). Therefore, *LRO1* was also deleted to ensure that no radiolabel was incorporated into TG via the PDAT pathway. As anticipated from the *in vivo* experiments, the Dga1p protein is the major determinant of oleoyl-CoA incorporation into TG. A residual level of TG synthesis persists in *dga1Δ* and *dga1Δ lro1Δ* microsomes, consistent with the *in vivo* assays. This activity is entirely accounted for by the Are2p enzyme; microsomes from triple *dga1Δ lro1Δ are2Δ* or quadruple *dga1Δ lro1Δ are1Δ are2Δ* mutants showed no oleoyl-CoA-dependent TG synthesis.

To define the acyl-CoA substrate specificity of Dga1p, microsomes were prepared from *lro1Δ are1Δ are2Δ* strains and assayed *in vitro* for the synthesis of TG from [1-3H]diacylglycerol and various acyl-CoA substrates (Table II). Oleoyl-CoA and palmitoyl-CoA were the preferred substrates while myristoyl-CoA, stearoyl-CoA, arachidonyl-CoA, and linoleoyl-CoA were used to a significantly lesser extent. By contrast, the Are1p and Are2p sterol esterification enzymes readily utilize linoleoyl-CoA as substrates while palmitoyl-CoA was barely incorporated (44). The reason for preferential incorporation of fatty acid species into TG or sterol ester is not known.

**The Evolution of Triglyceride Synthesis Reactions**—The lack of detectable DG esterification when the Dga1p, Lro1p, and Are2p reactions are absent suggests we have defined the compendium of TG biosynthetic reactions in this model. The use of acyl-CoA-dependent and -independent pathways for TG synthesis is conserved from yeast through plants (13, 41), although as yet, no Lro1p-like PDAT reaction has been detected in human cells. In an interesting example of convergent evolution, the O-acyltransferases (DGAT1p and Are2p) and the DGAT2 enzymes (such as DGAT2p and Dga1p) utilize the same substrates, but exhibit no conservation of primary structure. However, the reactions are distinct; *DGA1*, unlike the *ARE* genes, is transcriptionally up-regulated by conditions such as growth to saturation and nitrogen deprivation. Furthermore, the two acyl-CoA-dependent esterification reactions in *S. cerevisiae* exhibit differences in substrate recognition regarding the nature of the acyl chain. Both reactions readily utilize oleoyl-CoA; however, the yeast O-acyltransferases incorporate linoleoyl-CoA but not palmitoyl-CoA, while Dga1p is the converse. It is interesting to speculate that with regard to the alcohol substrate that yeast *ARE2* may represent the multi-substrate progenitor of its human orthologs ACAT1, ACAT2, and DGAT1, which subsequently developed more specific substrate specificities. Moreover, the existence of multiple DGA1 orthologs in humans suggests that the DGAT2 gene family has also been expanded and presumably specialized for undetermined functions (16). Whether DGAT1 and the members of the DGAT2 gene family are entirely responsible for DG

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**Fig. 3.** Dga1p and Lro1p contribute differentially to triglyceride biosynthesis. Normal (N), single, or multiple gene deletion haploids of the indicated genotype were grown in media containing 0.1% (w/v) oleate (YPTR0). Homozygous diploids of the indicated genotype were grown in sporulation media. Growth in the presence of [3H]glycerol proceeded for 24 h prior to extraction and resolution of triglyceride (Fig. 2A), diacylglycerol, and phospholipid (Fig. 2B) as described under "Experimental Procedures." Total dpm per TLC lane averaged 200,000–600,000 (YPTR0) and 50,000–300,000 (sporulation). The method of data expression and statistical significance are as described for Fig. 1. Asterisks denote statistically significant (p < 0.01) differences compared with normal strains.

**Fig. 4.** The yeast ergosterol O-acyltransferase, ARE2, mediates residual triglyceride synthesis. Mutant haploids of the indicated multiple deletion genotypes were grown to stationary phase, pulse-labeled with [3H]oleic acid and processed as described for Fig. 1. The data are presented as the percent (of total extractable lipids) incorporation into triglyceride, with standard deviations. Asterisks denote statistically significant (p < 0.01) differences compared with *dga1Δ lro1Δ* strains.
estervation in mammals remains to be determined.

What Is the Physiological Role of TG in Yeast?—These studies have allowed the production of a mutant strain (dga1Δ lro1Δ are2Δ) that lacks the ability to store energy as TG. Moreover, when we introduced a deletion mutation in the second sterol esterification enzyme, Are1p, into this strain, we created a strain devoid of neutral lipids. The sole growth phenotype observed was a prolonged lag phase of growth in YPD media relative to normal strains with no significant change in the exponential or stationary phases (data not shown). It is surprising that despite their evolutionary conservation, the neutral lipid biosynthetic enzymes appear to be dispensable for vegetative growth. This may be unique to the laboratory setting. In the wild, when nutrients are not readily available, storage may confer a selective advantage. We are now in the unique situation to explore how a eukaryotic cell copes with the loss of neutral lipid synthesis.

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