Appl Microbiol Biotechnol (2003) 61:289–299
DOI 10.1007/s00253-002-1212-4

MINI-REVIEW

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Triacylglycerol biosynthesis in yeast

Received: 23 September 2002 / Revised: 29 November 2002 / Accepted: 29 November 2002 / Published online: 29 January 2003

Abstract Triacylglycerol (TAG) is the major storage component for fatty acids, and thus for energy, in eukaryotic cells. In this mini-review, we describe recent progress that has been made with the yeast *Saccharomyces cerevisiae* in understanding formation of TAG and its cell biological role. Formation of TAG involves the synthesis of phosphatidic acid (PA) and diacylglycerol (DAG), two key intermediates of lipid metabolism. De novo formation of PA in yeast as in other types of cells can occur either through the glycerol-3-phosphate- or dihydroxyacetone phosphate-pathways—each named after its respective precursor. PA, formed in two steps of acylation, is converted to DAG by phosphatidate phosphatase. Acylation of DAG to yield TAG is catalyzed mainly by the two yeast proteins Dga1p and Lro1p, which utilize acyl-CoA or phosphatidylcholine, respectively, as acyl donors. In addition, minor alternative routes of DAG acylation appear to exist. Endoplasmic reticulum and lipid particles (LP), the TAG storage compartment in yeast, are the major sites of TAG synthesis. The interplay of these organelles, formation of LP, and enzymatic properties of enzymes catalyzing the synthesis of PA, DAG, and TAG in yeast are discussed in this communication.

Introduction

Within the last decade, biosynthesis of triacylglycerol (TAG) in many organisms has become an important field of research. Investigations with human cell lines and mammalian cells have demonstrated the influence of TAG accumulation on various diseases of the Western world such as obesity (Smith et al. 2000), diabetes (Song 2002) and arteriosclerosis (Taskinen 1997; Gotto 1998; Krauss 1998). Biotechnological interest in manipulation of lipid storage for agricultural purposes has resulted in advanced studies with plants (Hobbs et al. 1999; Routaboul et al. 1999; Zou et al. 1999; Dahlqvist et al. 2000). Research with the yeast *Saccharomyces cerevisiae*, an established and reliable eukaryotic model system with high similarity to other organisms, has helped to understand fundamental problems of TAG synthesis and to investigate the role of this lipid in various cell biological processes.

TAG is the molecule that can store energy at its highest concentration in eukaryotic cells and thus plays an essential role in energy storage and energy balance. Due to its low biological toxicity compared to free fatty acids (FFA), TAG provides an appropriate form of fatty acid storage. Therefore, TAG is utilized as fatty acid donor for membrane biogenesis, as has been shown with yeast, especially during depletion of fatty acids (Daum and Paltauf 1980; Leber et al. 1994). Moreover, unusual fatty acids that might have a toxic effect on the cell when incorporated into membranes can be stored in TAG and are excluded from membrane formation (Dodds 1995; Lehner and Kuksis 1996).

Synthesis of TAG in the yeast *S. cerevisiae* requires formation of its precursors phosphatidic acid (PA) and diacylglycerol (DAG). Two major de novo biosynthetic pathways that yield PA utilize either glycerol-3-phosphate (G-3-P) or dihydroxyacetone-phosphate (DHAP) as precursors (Fig. 1). G-3-P is acylated by G-3-P acyltransferase (GAT) at the *sn-1* position to form 1-acyl-G-3-P (lyso-phosphatidic acid, LPA), and then by 1-acyl-G-3-P acyltransferase (AGAT) in the *sn-2* position, yielding PA. Alternatively, DHAP is acylated at the *sn-1* position by DHAP acyltransferase (DHAPAT), and the product 1-acyl-DHAP formed is reduced by 1-acyl-DHAP reductase (ADR) to yield LPA, which is further acylated to PA by AGAT. PA can also be formed from phospholipids through the action of a phospholipase D, or by phosphorylation of DAG through DAG kinase (Fig. 1). Activation of PA with CTP by a CDP-DAG synthase leads to the formation of CDP-DAG, the precursor for phosphatidylinositol, phosphatidylglycerol, cardiolipin, phosphatidyl-

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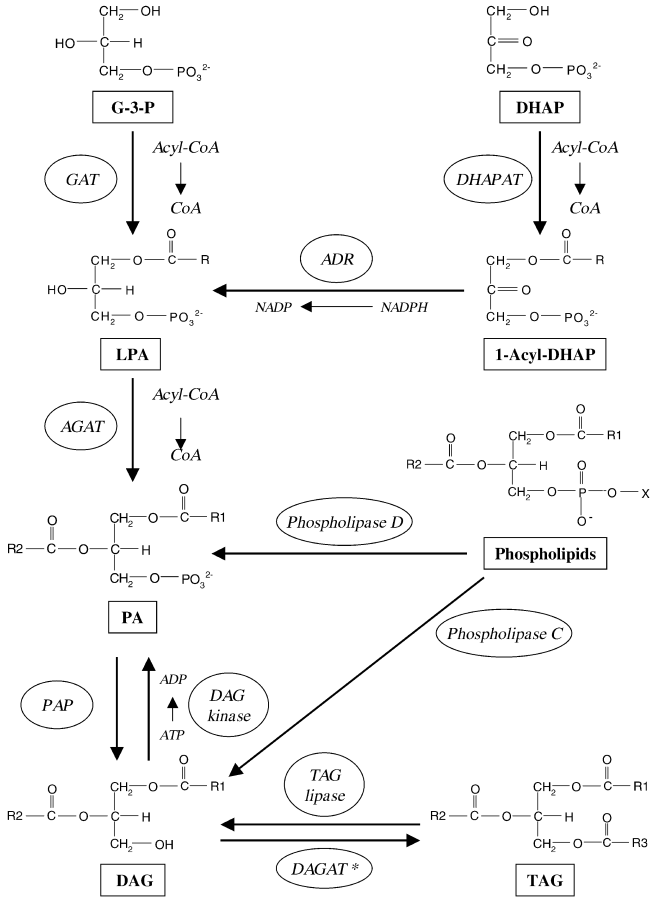

Fig. 1 Pathways for phosphatidic acid (PA) synthesis in the yeast *Saccharomyces cerevisiae*. *AGAT* 1-acyl-glycerol-3-phosphate acyltransferase, *ADR* 1-acyl-dihydroxyacetone-phosphate reductase, *DAG* diacylglycerol, *DAG kinase* diacylglycerol kinase, *DAGAT* diacylglycerol acyltransferase, *DHAPAT* dihydroxyacetone-phosphate acyltransferase, *DHAP* dihydroxyacetone-phosphate, *1-acyl-DHAP* 1-acyl-dihydroxyacetone-phosphate, *GAT* glycerol-3-phosphate acyltransferase, *G-3-P* glycerol-3-phosphate, *LPA* lysophosphatidic acid, PA phosphatidic acid, *PAP* phosphatidate phosphatase, *TAG* triacylglycerol, *TAG lipase* triacylglycerol lipase. Metabolites are enclosed in *rectangles*, enzymes in *ellipses*. *Co-substrates involved in DAGAT reactions are shown in Fig. 2

serine, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) (for reviews see Carman and Zeimetz 1996; Daum et al. 1998; Vance 1998; Carman and Henry 1999).

Table 1 Enzymes, genes, and metabolites involved in the synthesis of phosphatidic acid (PA) and triacylglycerol (TAG) in the yeast *Saccharomyces cerevisiae*. *1-acyl-DHAP* 1-Acyl-dihydroxyacetone-phosphate, *ADR* 1-acyl-dihydroxyacetone-phosphate reductase, *AGAT* 1-acyl-glycerol-3-phosphate acyltransferase, *DAG* diacylglycerol, *DAGAT* diacylglycerol acyltransferase, *DHAP* dihydroxyacetone-phosphate, *DHAPAT* dihydroxyacetone-phosphate acyltransferase, *GAT* glycerol-3-phosphate acyltransferase, *G-3-P* glycerol-3-phosphate, *LPA* lysophosphatidic acid, *PAP* phosphatidate phosphatase

Enzyme	Genes[a]	Substrate	Product
GAT	*GAT1*, *GAT2*	G-3-P	LPA
DHAPAT	*GAT1*, *GAT2*	DHAP	1-acyl-DHAP
ADR	*AYR1*	1-acyl-DHAP	LPA
AGAT	*SLC1*	LPA	PA
PAP	*DPP1*, *LPP1*	PA	DAG
DAGAT	*DGA1*, *LRO1*, *ARE1*, *ARE2*	DAG	TAG

[a] Only those genes characterized at the molecular level are listed

Dephosphorylation of PA by a phosphatidate phosphatase (PAP) yields DAG (Fig. 1), which is also formed from TAG by TAG lipases or from phospholipids through the action of a phospholipase C. DAG is a precursor for aminoglycerophospholipids via the Kennedy pathway and therefore a key intermediate in membrane lipid biosynthesis (for review see Kent 1995; Carman and Zeimetz 1996; Daum et al. 1998; Carman and Henry 1999; Voelker 2000), and substrate to diacylglycerol acyltransferases (DAGATs), which convert DAG to TAG using different acyl donors.

In this mini-review, we summarize present knowledge of TAG formation in the yeast *S. cerevisiae*. The enzymes catalyzing de novo synthesis of PA, DAG and TAG will be described with respect to their substrate specificity (Table 1) and their subcellular localization (Table 2). Interaction of organelles involved in TAG synthesis, and especially the formation of lipid particles (LP), the storage site for TAG and steryl esters (STE) (Leber et al. 1994), will be discussed.

PA synthesis in yeast

Acylation of G-3-P and DHAP

GAT activity in the yeast *S. cerevisiae* was first demonstrated by Christiansen (1978). That study, how-

Table 2 Localization of gene products involved in TAG synthesis in the yeast *S. cerevisiae*

Organelle	GAT	DHAPAT	ADR	AGAT	PAP[a]	DAGAT[b]
Endoplasmic reticulum	Gat1p, Gat2p (Sct1p)	Gat1p, Gat2p (Sct1p)	Ayr1p, unknown	Slc1p, unknown	Unknown (45 kDa; 104 kDa)	Lro1p, Dga1p,Are1p, Are2p
Lipid particles	Gat1p	Gat1p	Ayr1p	Slc1p	–	Dga1p
Mitochondria	–	Unknown	–	–	Unknown (45 kDa)	–
Cytosol	–	–	–	–	Unknown (75 kDa)	–
Vacuoles	–	–	–	–	Dpp1p	–

[a] Lpp1p was shown to be membrane-associated but was not assigned to a distinct cellular fraction
[b] Different acyl donors

ever, was restricted to the detection of enzymatic activity. Several years later, Tillman and Bell (1986) isolated yeast mutants defective in GAT activity using a colony radiographic screening technique. All mutants obtained during this study were similarly defective in GAT and DHAPAT activity. Tetrad analysis suggested mutations in a single nuclear gene encoding a polypeptide catalyzing both activities. This view came under scrutiny in investigations by Racenis et al. (1992) who showed that GAT and DHAPAT activities had different pH optima and different sensitivity against the sulfhydryl group reagent N-ethylmaleimide. This observation led the authors to conclude that the acylation of the two substrates is catalyzed by different enzymes.

In both studies described above, however, the authors had used only crude membrane fractions to measure enzymatic activities. Employing elaborate techniques of yeast cell fractionation developed in our laboratory, Zinser et al. (1991) and Athenstaedt and Daum (1997) revealed that the highest specific activity of GAT was detected in LP. This finding confirmed preliminary data presented earlier by Christiansen (1978) and suggested that LP of yeast are a major site of PA synthesis. LP of the mutant strain TTA1 (Tillman and Bell 1986), which harbors a point mutation in the *GAT* gene, were found to be essentially devoid of GAT activity in vitro, indicating that there is only one GAT in this compartment (Gat1p; Athenstaedt and Daum 1997). At that time, it came as a surprise that GAT activity in the microsomal fraction of this mutant was also significantly reduced, but a small amount of PA was still formed. This result demonstrated that (1) Gat1p is the only GAT of the LP fraction and also the major acyltransferase in the endoplasmic reticulum (ER), but (2) additional acyltransferase(s) contribute to PA synthesis via the G-3-P pathway in the latter organelle. Other subcellular fractions did not show any GAT activity. The obvious redundancy of enzymes also explained why the TTA1 mutant was viable.

To address the question of whether Gat1p is also responsible for the acylation of the precursor DHAP, Athenstaedt and Daum (1997) tested LP of TTA1 (hereafter referred to as *gat1*). Whereas the LP fraction of the wild type strain was able to form 1-acyl DHAP, LP of the *gat1* mutant did not show DHAPAT activity. This result demonstrated that Gat1p can acylate both G-3-P and DHAP and thus has dual substrate specificity. By analogy, microsomal fractions of the *gat1* mutant revealed similar reductions in both GAT and DHAPAT activity (Athenstaedt et al. 1999a). The precursor specificity of residual microsomal GAT activities could not be determined.

Whereas ER and LP appeared to be the only compartments harboring GAT activity, further investigations revealed that mitochondria are able to acylate the precursor DHAP (Athenstaedt et al. 1999a). Thus, an additional acyltransferase may be present in this organelle, preferring DHAP as the precursor and thus differing from Gat1p present in LP and ER and other putative acyltransferases located in the latter compartment.

Recently, Zheng and Zou (2001) discovered that the polypeptide encoded by the open reading frame (ORF) *YBL011w*, previously described as a choline transporter suppressor (*SCT1*) (Matsushita and Nikawa 1995), is involved in glycerolipid biosynthesis. Deletion of *YBL011w* resulted in a substantial decrease in total cellular GAT activity. A yeast strain bearing a deletion of the ORF *YKR067w*, which encodes a protein with 31% sequence identity to Sct1p, also exhibited a significant reduction in GAT activity. Molecular characterization of the genes revealed that a mis-sense mutation in *YKR067w* accounted for a defect in the GAT activity of the yeast mutant TTA1 previously described by Tillman and Bell (1986). Zheng and Zou (2001) thus concluded that the gene product of *YKR067w* catalyzes the LP GAT activity and was therefore Gat1p, whereas the gene product encoded by *YBL011w* was named Gat2p. Both Gat1p and Gat2p were able to utilize G-3-P and DHAP as substrates and thus exhibited a dual substrate-specific *sn-1*-acyltransferase activity. Whereas Gat1p effectively utilized a broad range of fatty acids as acyl donors, Gat2p displayed a preference towards 16-carbon fatty acids. The *gat1* and *gat2* single mutant strains showed a decreased PA pool and an increased phosphatidylserine to phosphatidylinositol ratio but still grew like wild type. A *gat1 gat2* double mutation, however, is lethal to yeast cells (Zheng and Zou 2001). Most recently, Zaremberg and McMaster (2002) demonstrated that inactivation of *GAT1* increased TAG synthesis by 50%, while TAG synthesis was decreased by 50% in yeast lacking *GAT2*. Thus, acylation through Gat2p is the major route for the downstream synthesis of TAG. Since Gat2p is localized to microsomes and exhibits dual substrate specificity, the putative mitochondrial DHAPAT described by Athenstaedt et al. (1999a) still awaits identification.

The link between the DHAP and G-3-P pathways

To channel 1-acyl-DHAP into the G-3-P pathway, conversion of 1-acyl-DHAP to LPA by ADR is required. The presence of an NADPH-dependent ADR activity in yeast was first reported by Racenis et al. (1992) but neither the gene nor the gene product were characterized at the molecular level at that time. Enzymatic analysis of subcellular fractions (Athenstaedt and Daum 2000) led to localization of ADR activity to LP and ER, whereas this enzyme appeared to be absent in mitochondria. Systematic studies of the most prominent LP proteins identified by mass spectrometry (Athenstaedt et al. 1999b) led to the identification of an ADR at the molecular level. This polypeptide, encoded by the ORF *YIL124w* (*AYR1*), has a molecular weight of 33 kDa and is the only enzyme catalyzing the reduction of 1-acyl DHAP in LP. Lack of transmembrane domains and the presence of only few hydrophobic domains in this polypeptide are in line with characteristic features of LP proteins (Athenstaedt et al. 1999b). LP of an *ayr1* single mutant were completely devoid of ADR activity, whereas in the ER the deletion of

AYR1 only caused a decrease to 30% of wild type activity. This result demonstrated that (1) Ayr1p is distributed between two different cell compartments, and (2) additional enzymes for the conversion of 1-acyl DHAP to LPA must be present in the microsomal fraction. The latter conclusion was supported by the observation that an *ayr1* mutant strain grows like wild type and has a normal lipid composition. Heterologous expression of Ayr1p in *Escherichia coli* resulted in increased ADR activity in the prokaryote, confirming that *YIL124w* is the structural gene of the enzyme and not a regulatory or auxiliary component required for reduction of 1-acyl DHAP (Athenstaedt and Daum 2000). During phenotypic analysis it was shown that spores bearing the *ayr1* defect failed to germinate. A specific role of Ayr1p in sporulation, however, could not be demonstrated. Recently, *AYR1* was found to be involved in dehydroepiandrosterone (DHEA) metabolism in *S. cerevisiae* (Vico et al. 2002). Disruption of *AYR1* abolished the reduction of DHEA to 3β,17β-androstenediol indicating that *Ayr1p* harbors the major 17β-hydroxysteroid dehydrogenase activity of yeast. Furthermore, Han et al. (2002) postulated a 3-ketoreductase activity of Ayr1p and its involvement in fatty acid elongation. Thus, Ayr1p appears to exhibit broad substrate specificity.

Acylation of LPA

In a screen searching for suppressors of a genetic defect in sphingolipid long chain base biosynthesis the suppressor gene *SLC1* (sphingolipid compensation 1) was isolated and characterized (Nagiec et al. 1993). The predicted protein sequence of Slc1p was shown to be homologous to the AGAT of *E. coli* encoded by the *plsC* gene. Successful complementation of the growth defect of an *E. coli plsC* mutant by heterologous expression of *SCL1* confirmed that Slc1p has fatty acyltransferase activity. Localization studies with the *slc1* yeast mutant YMN5 (Nagiec et al. 1993) by Athenstaedt and Daum (1997) revealed that Slc1p is the only AGAT in LP, although Slc1p is also present in the ER. Localization of Slc1p to LP was also confirmed by two-dimensional gel electrophoresis of LP proteins isolated from *slc1* and wild type cells. Residual AGAT activity independent of Slc1p was detected in the ER of a *slc1* mutant indicating the presence of additional AGATs in this compartment. Such proteins, however, have not yet been identified at the molecular level.

Fatty acid substrate specificity of Slc1p was determined by Zou et al. (1997). Slc1p was shown to exhibit *sn-2* acyltransferase activity in vitro with a broad range of acyl-CoA thioesters, including 18:1-, 22:1-, and 24:0-CoAs. In transgenic plants harboring the yeast *SLC1* gene, a substantial increase in the seed oil content and in overall proportions and amounts of very long chain fatty acids in seed TAG were discovered. Moreover, incorporation of very long chain fatty acids at the *sn-2* position of TAG was increased. Thus, Slc1p can be used to modify total fatty acid content and composition to alter the stereospecific acyl chain distribution in seed TAG.

Bypass pathways of PA formation

Besides de novo biosynthesis, PA can be formed by alternative routes that may be important to compensate defects in the de novo synthesis of PA (see Fig. 1). Hydrolysis of glycerophospholipids through the action of phospholipase D and phosphorylation of DAG by DAG kinase also lead to the formation of PA. DAG is the deacylation product derived from TAG by the action of TAG lipases, or formed by hydrolysis of glycerophospholipids through phospholipase C. These pathways, however, will not be discussed in this mini-review. The reader is referred to recent reviews by Munnik and Musgrave (2001), Liscovitch et al. (2000), Rebecchi and Pentyala (2000), Huijbregts et al. (2000), and Van Heusden et al. (1998).

Hydrolysis of PA leads to DAG formation

The enzyme catalyzing dephosphorylation of PA yielding DAG and P_i is phosphatidate phosphatase (3-*sn*-phosphatidate phosphohydrolase; PAP). Hosaka and Yamashita (1984a) were the first to postulate PAP activity in the yeast *S. cerevisiae*. High specificity of the enzyme for PA was shown in the cytosol and in membrane fractions. The membrane-associated activity of PAP was equally distributed between microsomes and mitochondria (Hosaka and Yamashita 1984a; Morlock et al. 1988). Two forms of PAP, a 104 kDa and a 45 kDa species, were found in the membrane fractions. Whereas the 45-kDa protein was localized to both microsomes and mitochondria, the 104 kDa protein was restricted to the ER. Both enzymes are activated in the presence of Mg^{2+} and inhibited by N-ethylmaleimide, phenylglyoxal and propranolol, but only the 104 kDa protein is activated by CDP-DAG, cardiolipin and phosphatidylinositol (Wu and Carman 1996). Both PAPs require Triton X-100 for maximum activity in vitro and are unstable at temperatures above 30°C (Morlock et al. 1988; Lin and Carman 1989). The cytosolic form of PAP, with a molecular mass of approximately 75 kDa, is activated by Mg^{2+}, whereas N-ethylmaleimide, CDP-DAG, DAG and TAG inhibit this enzyme (Hosaka and Yamashita 1984a). A null mutant of a putative PAP found by sequence alignment has an abnormal shape and abnormal cytokinesis during cell division (Katagiri and Shinozaki 1998). These data suggested that yeast PAP is possibly involved in cell growth and cytokinesis.

Regulation of yeast PAPs is complex and depends on various factors. Morlock et al. (1988) postulated that PAP activity is increased when yeast cells are supplemented with inositol and when the cells enter the stationary growth phase. Addition of inositol to the growth medium, however, induced only the 45 kDa PAP. The expression

of the 104-kDa enzyme was not affected by inositol, whereas both forms of PAP increased in the stationary phase. Enzyme activity of PAP was not regulated in inositol biosynthesis regulatory mutants. Hosaka and Yamashita (1984b) postulated that PAP plays an important role in the regulation of TAG synthesis in *S. cerevisiae* because the increase in PAP activity in the stationary phase correlated with the accumulation of TAG. This result is in line with the finding that increased activities in stationary cells of several proteins involved in PA synthesis, e.g., GAT, DHAPAT and ADR, led to enhanced formation of TAG and simultaneously to reduced synthesis of phospholipids. PAP activity is also regulated by lipids, nucleotides and by phosphorylation (Carman and Zeimetz 1996) whereas serine, ethanolamine, and choline did not significantly affect the PAP activity of cells grown in the absence or presence of inositol (Morlock et al. 1988). For detailed description of these aspects the reader is referred to reviews by Carman (1997) and Kocsis and Weselake (1996).

Diacylglycerol pyrophosphate (DGPP) phosphatase is an enzyme harboring a PAP activity that is Mg^{2+}-independent, N-ethylmaleimide-insensitive and distinct from the Mg^{2+}-dependent and N-ethylmaleimide-sensitive form of PAP (Wu et al. 1996). The two yeast genes *LPP1* and *DPP1*, which are homologous to the mammalian PAP2, were shown to catalyze DGPP phosphatase activity. Dpp1p is a membrane-associated enzyme that catalyzes first removal of the β-phosphate from DGPP to form PA and then the phosphate from PA to form DAG. The preferred substrate of Dpp1p is DGPP, but PA can also be utilized as a substrate to a certain extent. The enzyme requires Triton X-100 for maximum activity in vitro and is inhibited by divalent cations, NaF, and pyrophosphate (Wu et al. 1996). Recent investigations revealing inhibition of Dpp1 by zinc (Lyons et al. 2000; Yuan 2000) were confirmed by Han et al. (2001), who demonstrated formation of DGPP-zinc complexes. Moreover, the authors showed that Dpp1p is localized to the vacuolar membranes.

The *LPP1* (lipid phosphate phosphatase) gene encodes a protein containing a phosphatase sequence motif found in DGPP phosphatase and in the mouse Mg^{2+}-independent PA phosphatase (Toke et al. 1998). Lpp1p is associated with the membrane fraction of the cell. This gene product exhibited LPA phosphatase and DGPP phosphatase activities (Furneisen and Carman 2000) with a substrate preference in the order PA>LPA>DGPP. The enzymatic properties of Lpp1p were significantly different from Dpp1p and similar to mammalian proteins, especially with respect to the inhibition of Lpp1p by N-ethylmaleimide and phenylglyoxal. In addition, the divalent cations Mn^{2+}, Co^{2+}, and Ca^{2+}, NaF, heavy metals and propranolol, inhibited the PA phosphatase, DGPP phosphatase and LPA phosphatase activities of the enzyme (Furneisen and Carman 2000). The single mutants *dpp1* and *lpp1* and the double mutant *lpp1 dpp1* were viable and did not exhibit obvious growth defects. Biochemical analysis of these strains showed that the *LPP1* and *DPP1* gene products encoded nearly the complete Mg^{2+}-independent PA phosphatase and LPA phosphatase activities and the entire DGPP phosphatase activity in *S. cerevisiae*. Moreover, analysis of the mutants showed that the gene products of *LPP1* and *DPP1* are involved in the regulation of phospholipid metabolism and influence the cellular levels of phosphatidylinositol and PA (Toke et al. 1998). Faulkner et al. (1999) postulated that isoprenoid phosphates are also substrates for Lpp1p and Dpp1p. Single deletions of these genes caused a decrease of about 25% and 75% of Mg^{2+} independent hydrolysis of several isoprenoid phosphates, respectively, whereas in *lpp1dpp1* double mutants Mg^{2+} independent hydrolysis of dolichyl phosphate, dolichyl pyrophosphate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate was essentially lost.

TAG biosynthesis in yeast: acylation of DAG

Dga1p and Lro1p catalyze two different pathways of TAG formation

DAG formed from PA by a PAP or by alternative pathways (see above) is converted to TAG in another acylation step utilizing different acyl donors. The most prominent enzyme catalyzing this step is acyl-CoA:diacylglycerol acyltransferase (DAGAT; EC 2.3.1.20), which was shown to form TAG from DAG and acyl-CoAs as substrates (for a review see Lehner and Kuksis 1996). Christiansen (1978, 1979) demonstrated localization of yeast DAGAT in LP using endogenous 1,2-diacylglycerol as substrate in vitro. Although genes of the DGAT1 family encoding DAGATs homologous to acyl-CoA:cholesterol acyltransferase (ACAT) were identified in plants (Hobbs et al. 1999; Routaboul et al. 1999; Zou et al. 1999) and in mice (Cases et al. 1998), the yeast enzyme catalyzing this reaction was not characterized at the molecular level for a long time. Most recently, a DAGAT unrelated to the DGAT1 family was identified from the oleaginous fungus *Mortierella ramanniana* (Cases et al. 2001; Lardizabal et al. 2001). This result led to the identification of a *S. cerevisiae* DAGAT, which showed a 44% overall amino acid sequence homology to the enzyme from *M. ramanniana*, but was unrelated to ACATs and DAGATs of the DGAT1 family. The yeast ORF *YOR245c* was found to encode the yeast DAGAT (hereafter Dga1p), a protein with a molecular mass of approximately 47.7 kDa. Heterologous expression of *YOR245c* in insect cells led to a significant increase of DAGAT activity in the host (Cases et al. 2001; Lardizabal et al. 2001), and disruption of *YOR245c* in yeast caused reduced TAG synthesis in vitro and a decrease in the cellular amount of TAG (Oelkers et al. 2002; Sandager et al. 2002; Sorger and Daum 2002). Due to its structural features, Dga1p was suggested to belong to a new DAGAT gene family called DGAT2. Localization studies by Sorger and Daum (2002) using a newly established in vitro assay containing ^{14}C-labeled DAG and acyl-CoA as

substrates revealed a 70- to 90-fold enrichment of DAGAT activity in LP over the homogenate, but also a 2- to 3-fold enrichment in microsomal fractions. The activity of the enzyme was strongly dependent on the presence of K^+ and Mg^{2+}. Whereas the specific DAGAT activity in microsomes from a *dga1* single mutant strain was only slightly decreased compared to wild type, LP of this mutant showed an activity reduced to 5% of the wild type control. These data indicated that (1) Dga1p is the major, probably the only, acyl-CoA dependent DAGAT in LP, (2) Dga1p is also localized to the ER, and (3) additional enzymes catalyzing acyl-CoA dependent DAGAT activity must be present in the latter compartment. The *dga1* deletion strain formed regular LP and exhibited normal growth properties, confirming the existence of additional TAG synthases (Sorger and Daum 2002).

Computational analysis led to the identification of another TAG synthesis pathway catalyzed by the gene product of *YNR008w*. Sequence alignments with human lecithin cholesterol acyltransferase (LCAT) revealed that *YNR008w* (*LRO1*; LCAT-related ORF) is the only gene in yeast showing a distinct sequence similarity with 27% overall identity and conservation of Ser^{181} and Asp^{345}, two members of the LCAT catalytic triad. Lro1p mediates esterification of DAG using the *sn-2* acyl group of PC as acyl donor and thus exhibits phospholipid:diacylglycerol acyltransferase (PDAT) activity. In vitro assays revealed that wild type microsomes can utilize the *sn-2* acyl-group of PC for acylation of DAG whereas microsomes of the *lro1* mutant lack this activity completely. In addition, experiments with yeast cells overexpressing *LRO1* resulted in an increased TAG content, confirming the involvement of *LRO1* in TAG formation (Dahlqvist et al. 2000; Oelkers et al. 2000). Acylation of DAG using acyl-CoA as substrate, however, was unchanged in an *lro1* mutant strain (Oelkers et al. 2000; Sorger and Daum 2002) indicating that Dga1p and Lro1p catalyze different reactions of TAG formation. Moreover, Lro1p activity was not detected in LP as shown previously for Dga1p, but exclusively in the ER (D. Sorger and G. Daum, unpublished data).

Contribution of Dga1p and Lro1p to TAG synthesis

The contribution of Dga1p to TAG synthesis in yeast is still a matter of debate. Whereas Sorger and Daum (2002) postulated that the amount of TAG in a *dga1* mutant is reduced 30% compared to wild type, Sandager et al. (2002) proposed Dga1p to be responsible for 87% of TAG formed in yeast. These different results might be due to different wild type backgrounds. On the other hand, the contribution of Dga1p to cellular TAG synthesis is growth phase dependent. Oelkers et al. (2002) demonstrated that cells of the *dga1* mutant harvested in the logarithmic phase showed a 25% reduction in the amount of TAG, whereas cells grown to the stationary phase exhibited a 50% reduction. It has been shown previously that changing environmental conditions during cell growth also causes alterations in TAG synthesis (Taylor and Parks 1979; Hosaka and Yamashita 1984a).

An *lro1* deletion strain grown to the logarithmic phase exhibited 65% decreased incorporation of oleate into TAG, a 75% decrease in glycerol incorporation into TAG, and a significantly reduced amount of TAG as compared to wild type (Oelkers et al. 2000). A strain harboring *LRO1* as the only gene involved in TAG synthesis contained 38% of the amount of TAG found in wild type (Sandager et al. 2002). This result is in line with findings from our own laboratory (Daum et al. 1999; Sorger and Daum, 2002) but differs from data presented by Dahlqvist et al. (2000), who showed that the amount of TAG was only slightly reduced in *lro1*. Again, the influence of different growth phases on TAG synthesis has to be considered. Wild type and *lro1* grown to the stationary phase did not show a difference in oleate incorporation into TAG indicating that *LRO1* effects TAG formation only in the logarithmic phase. In contrast, *DGA1* is mainly involved in TAG synthesis during the stationary phase (Oelkers et al. 2002). These data correlate with (1) the finding that expression of *LRO1* does not coincide with the major phase for TAG accumulation during late stationary phase (Dahlqvist et al. 2000), and (2) transcriptional up-regulation of *DGA1* relative to *LRO1* during the diauxic shift and stationary growth (Gasch et al. 2000). Independent of the growth phase, however, a 97% reduction in TAG synthesis in a *dga1 lro1* double mutant was reported by Oelkers et al. (2002), whereas Sorger and Daum (2002) postulated that *dga1 lro1* cells grown to the late logarithmic growth phase still retained 20% of the amount of TAG in wild type. Again, differences might be due to the different wild type backgrounds used in these studies. Similar to *dga1* and *lro1* single mutants, the *dga1lro1* double mutant grows like wild type and is able to form LP, although with a significant reduction of number and size (Oelkers et al. 2002). These results indicate that (1) Dga1p and Lro1p are the major contributors to TAG acylation in yeast, but (2) alternative pathways for TAG synthesis exist.

Alternative routes of DAG acylation

In the yeast *S. cerevisiae*, *ARE1* and *ARE2* (ACAT related enzymes) are the only genes catalyzing STE synthesis. Both isoenzymes have acyl-CoA:sterol acyltransferase (ASAT) activity (Yang et al. 1996; Yu et al. 1996) and share 16% and 17% amino acid identity, respectively, with human ACAT (Yu et al. 1996). DAGATs and ACATs catalyzing similar reactions are approximately 20% identical in plants (Hobbs et al. 1999; Zou et al. 1999) and in mice (Cases et al. 1998) with some highly conserved amino acid motifs especially in their C-termini. This similarity led to the speculation that Are1p and Are2p are probably involved not only in the acylation of sterols but also of DAG. The relative contribution of these genes to TAG formation in yeast, however, is still a matter of debate. Using a set of triple deletion strains in

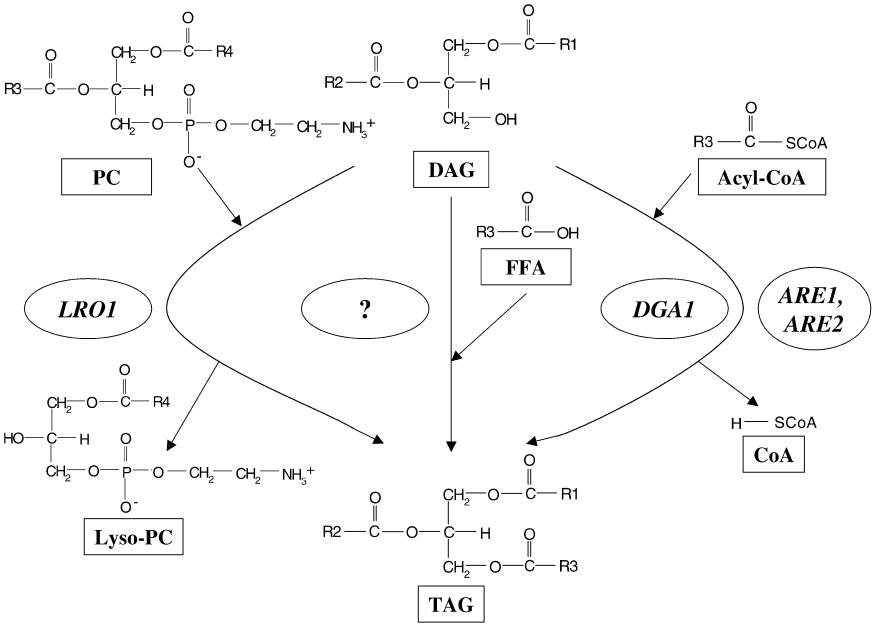

Fig. 2 Pathways for TAG synthesis in the yeast *S. cerevisiae*. FFA Free fatty acid, PC phosphatidylcholine. Metabolites are enclosed in *rectangles*, genes in *ellipses*. The *question mark* indicates involvement of a so far unknown gene in the reaction

which each strain expresses only one of the four genes responsible for neutral lipid synthesis in yeast (*DGA1*, *LRO1*, *ARE1* or *ARE2*), Sandager et al. (2002) discovered DAGAT activities of Are1p and Are2p in addition to the major TAG synthases Dga1p and Lro1p, although at a very low level. The finding that both ACAT-related genes in yeast harbor DAGAT activity does not correlate with previous data postulating that only Are1p is involved in synthesis of TAG whereas Are2p is restricted to sterols as substrates (Sandager et al. 2000). This finding is in line with recent results from our laboratory (D. Sorger, unpublished results) indicating that only Are1p has DAGAT activity. In contrast, Oelkers et al. (2002) argued that Are2p is responsible for the residual TAG formation when *DGA1* and *LRO1* are deleted. Thus, the contribution of Are-proteins to TAG formation remains unclear. In a quadruple mutant lacking all four genes (*DGA1*, *LRO1*, *ARE1* and *ARE2*) no storage lipid and, as a consequence, no LP (which consist of 51% TAG and 44% STE in wild type; Leber et al. 1994), were detected. This result indicated that Dga1p, Lro1p, Are1p, and Are2p are the only proteins catalyzing the final steps in neutral lipid synthesis in yeast (Oelkers et al. 2002; Sandager et al. 2002). The quadruple mutant is still viable and has normal growth properties except the reduction of the final density to 75% of wild type. These data indicate that storage lipid synthesis is not essential in yeast. The quadruple mutant, however, showed a reduced viability after nitrogen starvation suggesting that the ability to accumulate storage lipids is important for long term survival in stationary phase (Sandager et al. 2002).

Another TAG synthase activity different from Dga1p and Lro1p was observed in LP and microsomal fractions of *S. cerevisiae* (Sorger and Daum 2002). Experiments performed with radioactively labeled DAG and FFA as co-substrates showed a moderate but significant TAG synthase activity, which was shown to be independent of acyl-CoA formation catalyzed by the fatty acid activator proteins Faa1p to Faa4p. Incorporation of radioactively labeled FFA into complex lipids of *S. cerevisiae* was shown previously by Wagner and Paltauf (1994). FFA were preferentially incorporated into phospholipids in vivo, suggesting a phospholipase A_2-dependent deacylation-reacylation mechanism. In addition, FFA were incorporated into TAG, which led to the speculation that a similar mechanism might be involved in the formation of TAG. However, genes and gene products involved in such a reaction are still unknown. In summary, different pathways seem to be involved in acylation of DAG in *S. cerevisiae* (Fig. 2).

In contrast to *S. cerevisiae*, in which TAG formation appears to be distributed between LP and microsomes, TAG biosynthesis in the oleaginous yeast *Rhodotorula glutinis* only partly occurs in the membrane fraction. Approximately 60% of total TAG synthase activity in this microorganism is associated with the cytosolic fraction (Gangar et al. 2001a). A cytosolic 10 S multienzyme complex catalyzing TAG biosynthesis during exponential growth has been isolated, purified and characterized. This complex, which contains LPA acyltransferase, PA phosphatase, DAG acyltransferase, acyl-acyl carrier protein (ACP) synthetase, and ACP, is stabilized by protein-protein interactions and can incorporate acyl-CoA as well as FFA into TAG and its biosynthetic intermediates (Gangar et al. 2001a). FFAs are activated by a 35 kDa protein harboring acyl-ACP synthetase activity that catalyzes the ATP-dependent ligation of fatty acid with ACP to form acyl-ACP (Gangar et al. 2001b). A dramatic reduction of growth rate and TAG production was observed in the non-lethal mutants *tag1* and *tag2* defective in LPA and DAG acyltransferase (Gangar et al. 2002). In the *tag1* mutant, the amount of TAG was

decreased by 90%, and the formation of PA and DAG was also reduced. In contrast, in a *tag2* mutant, only the TAG content was decreased by 86% compared to wild type. The activity of the membrane-bound isoforms of TAG biosynthetic enzymes remained unaltered in both mutant strains.

Substrate specificity of DAG-acylating proteins

LRO1, the yeast homolog of human LCAT, is known to utilize the *sn*-2 acyl group of PC for acylation of DAG. Dahlqvist et al. (2000) demonstrated that properties of the substrate have a strong influence on this acylation step. The authors discovered that dioleoyl-PE is a 4-fold better substrate than dioleoyl-PC. Furthermore, a ricinoleoyl group at the *sn-2* position of PC is 2.5 times more efficiently incorporated into TAG than PC with an oleoyl group in this position. Moreover, DAG with a ricinoleoyl or a vernoloyl group is a better acyl acceptor than dioleoyl DAG. These data indicate that the rate of acyl transfer strongly depends on the head group of the acyl donor, the acyl species that is transferred and acyl chains in the acceptor DAG molecule. Interestingly, ergosterol is not a substrate for Lro1p although *LRO1* shows high homology to human LCAT.

The acyl-CoA-dependent Dga1p, Are1p and Are2p have different substrate specificities in vitro (Oelkers et al. 2002). Dga1p seems to prefer incorporation of oleoyl-CoA and palmitoyl-CoA, while myristoyl-CoA, stearoyl-CoA, arachidonyl-CoA and linoleoyl-CoA are not as efficiently used as substrates. Are1p and Are2p readily utilize linoleoyl-CoA as substrates while palmitoyl-CoA is barely incorporated (Yang et al. 1997). Zweytick et al. (2000b) demonstrated that *are1* and *are2* single mutants contain STE with a fatty acid composition resembling that of wild type. However, the sterol composition of STE from Are2p and Are1p was different. Whereas Are2p prefers ergosterol as a substrate in vivo rather than its precursors, Are1p esterifies ergosterol and various steryl precursors in vivo with similar efficiency, showing a slight preference for lanosterol. Strains lacking *DGA1* exhibit an altered fatty acid composition in TAG, STE and total lipids (Sandager et al. 2002). Whereas vaccenic acid and palmitoleic acid accumulate in these strains, the amount of palmitic acid is reduced. The reason for this effect might be the decreased TAG synthesis in these strains.

Interplay of organelles involved in TAG synthesis

The yeast *S. cerevisiae* exhibits redundant biosynthetic systems of PA, DAG, and TAG formation. As shown in Table 2, each step of TAG synthesis is catalyzed by at least two gene products with different properties. Isoenzymes are different with respect to their substrate specificities and growth-dependent expression levels. In addition, various organelles are involved in TAG synthesis of the yeast *S. cerevisiae*. The ER, which contains the complete set of TAG synthesizing enzymes, is the major site of TAG formation. However, LP are also able to form TAG autonomously, because Gat1p, Ayr1p, and Slc1p, identified as components of LP (Athenstaedt and Daum 1997; Athenstaedt et al. 1999a), catalyze PA formation. PAP, partly localized to the cytosol, may have access to the surface of LP, converting PA into DAG. Furthermore, Dga1p catalyzing DAG acylation is a LP protein (Sorger and Daum 2002). In addition, the acyl-CoA forming fatty acid activators Faa1p and Faa4p, which synthesize the co-substrates for the acylation of DAG through Dga1p, are also present in LP (Athenstaedt et al. 1999b). Since all LP proteins are located on the surface monolayer of these droplets, TAG seems to be formed in situ in or close to this monolayer membrane. The mechanism of TAG assembly into the core of LP is unknown. Self-assembly of neutral lipids or protein-catalyzed incorporation may be considered as possible processes.

Table 2 shows that the ER and LP share a set of proteins and therefore seem to be highly related compartments. All LP proteins identified so far are part of lipid-biosynthetic pathways that, at least in part, occur also in the ER. The fact that most LP proteins lack transmembrane domains (Athenstaedt et al. 1999b) led to the assumption that these proteins may have left the bilayer environment of the ER during formation of LP. Thus, the ER is generally regarded as the origin of LP, which may most likely be formed in a budding process (for a review see Zweytick et al. 2000a). LP biogenesis may start with the synthesis of TAG and STE in the membrane bilayer of the ER, leading to accumulation of neutral lipid droplets between the two envelopes. LP containing the cytosolic envelope of the ER membrane, a phospholipid monolayer, will then be newly formed. Membrane domains containing proteins of TAG metabolism lacking transmembrane domains may be preferentially incorporated into nascent droplets. Finally, LP bud off upon reaching a certain size. This model is supported by the finding that nascent oil bodies of plants were found between the envelopes of the ER (Lacey et al. 1999). Other models of LP formation such as targeting of components to pre-existing neutral lipid cores, vesicle flux, or spontaneous clustering of components, appear to be less likely (Zweytick et al. 2000a). Nevertheless, assembly of proteins with LP appears to depend on certain properties of the polypeptides. Recently, it was shown in our laboratory (H. Müllner, unpublished results) that hydrophobic domains near the C-terminus are required for embedding LP proteins into the surface monolayer membrane of this compartment.

As argued in the previous paragraph, TAG stored in LP may be synthesized either (1) in the ER prior to LP formation, or (2) on the surface of LP with subsequent translocation into the core of the droplet. Recent investigations in our laboratory revealed that, in addition to TAG synthesizing enzymes, TAG lipases-enzymes which catalyze hydrolysis and mobilization of TAG—are also present in LP (K. Athenstaedt, unpublished results). Thus,

accumulation of TAG in LP and mobilization of TAG from this site may be coordinately regulated by the requirement of fatty acids for cellular processes.

Besides the major TAG-synthesizing compartments ER and LP, other organelles such as mitochondria, cytosol, and vacuoles are also involved in TAG formation in yeast. As shown in Table 2, mitochondria contain a DHAPAT that is different from Gat1p and Gat2p but has not yet been identified at the molecular level (Athenstaedt et al. 1999a). Due to the fact that mitochondria lack ADR activity, the question arises as to how 1-acyl DHAP formed by the putative mitochondrial DHAPAT migrates to the site of reduction. Three mechanisms might explain this process: (1) simple diffusion from mitochondria to the ER because of the solubility of 1-acyl DHAP in water, (2) transport by means of carrier proteins or vesicles, or (3) membrane contact, probably through so-called mitochondria associated membranes (MAM) (Gaigg et al. 1995; Achleitner et al. 1999). Thus, 1-acyl DHAP may be further converted to PA in the ER and/or in MAM. CDP-DAG formed in the subsequent step in MAM or in mitochondria (Kuchler et al. 1986; Kelley and Carman 1987; Zinser et al. 1991) might serve as a preferred substrate for cardiolipin synthesis in mitochondria. This hypothesis is confirmed by the finding that the DHAP pathway contributes more to cardiolipin synthesis than the G-3-P pathway (Athenstaedt et al. 1999a). Thus, one reason for the redundancy of enzymes and their different localization could be the requirement of distinct pools of key intermediates. It should also be mentioned that LPA, PA, and DAG serve not only as precursors for glycerolipid and TAG formation, but also as signaling molecules in different organelles. Thus, each organelle may harbor certain enzymes that guarantee a local and quick response to the requirement of certain lipid intermediates.

Conclusions and future perspectives

Although many enzymes involved in TAG synthesis in the yeast *S. cerevisiae* have been identified and characterized at the molecular level during the last few years, several questions regarding the cell biology of TAG formation and utilization remain open. The presence of enzymes with overlapping function in the PA biosynthetic pathway raises the question of their physiological role. Some putative proteins, such as the mitochondrial DHAPAT, the microsomal ADR and a microsomal AGAT, and their corresponding genes are still not known. The relevance of different cellular pools of PA and its intermediates needs to be demonstrated. Understanding the cellular role of the two different routes of TAG formation governed by Dga1p and Lro1p will be another challenge for future research. Side branches of TAG biosynthesis such as fatty acid exchange or acyl-CoA-independent incorporation of fatty acids also require elucidation.

Another aspect related to TAG synthesis is the biogenesis of LP, where TAG is stored. Regulatory aspects of TAG synthesis and formation of neutral lipid depots in LP are far from clear. To increase our knowledge about mobilization of TAG and STE from LP by TAG lipases and STE hydrolases, respectively, requires identification of these proteins at the molecular level. Additional, as yet unknown, factors may also be involved in this process. Using the yeast *S. cerevisiae* as a eukaryotic model system will, however, be an appropriate approach to address these questions. The use of mutant strains, the availability of appropriate methods of organelle analysis, the design of novel screening strategies to search for missing genes, and the application of modern methods of lipid analysis will form the basis for a better understanding of TAG synthesis in yeast.

Acknowledgements Studies in our laboratories concerning TAG synthesis in yeast were financially supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (project 15141) and the Austrian Ministry of Education, Science and Culture (project AUSTROFAN).

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