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Review

Dynamics of neutral lipid storage in yeast★✪

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Since energy storage is a basic metabolic process, the synthesis of neutral lipids occurs in all kingdoms of life. The yeast, *Saccharomyces cerevisiae*, widely accepted as a model eukaryotic cell, contains two classes of neutral lipids, namely steryl esters and triacylglycerols. Triacylglycerols are synthesized through two pathways governed by the acyl-CoA diacylglycerol acyltransferase Dga1p and the phospholipid diacylglycerol acyltransferase Lro1p, respectively. Steryl esters are formed by the two steryl ester synthases Are1p and Are2p, two enzymes with overlapping function which also catalyze triacylglycerol formation, although to a minor extent. Storage of neutral lipids is tightly linked to the biogenesis of so called lipid particles. The role of this compartment in lipid homeostasis and its interplay with other organelles involved in neutral lipid dynamics, especially the endoplasmic reticulum and the

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Abbreviations: ACAT, acyl-CoA cholesteryl acyltransferase; ACP, acyl carrier protein; ADR, 1-acyl-DHAP reductase; AGAT, 1-acyl-G-3-P acyltransferase; ALA, δ-aminolevulinic acid; DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; DHAPAT, DHAP acyltransferase; ER, endoplasmic reticulum; GAT, G-3-P acyltransferase; GFP, green fluorescent protein; G-3-P, glycerol-3-phosphate; HSL, hormone-sensitive lipase; LCAT, lecithin cholesteryl acyltransferase; LPA, lyso-phosphatidic acid; LPL, lipoprotein lipase; MAG, monoacylglycerol; PAP, phosphatidate phosphatase; PDAT, phospholipid diacylglycerol acyltransferase; STE, steryl esters; TAG, triacylglycerol; VLDL, very low density lipoprotein.

plasma membrane, are subject of current investigations. In contrast to neutral lipid formation, mobilization of triacylglycerols and steryl esters in yeast are less characterized at the molecular level. Only recently, the triacylglycerol lipase Tgl3p was identified as the first yeast enzyme of this kind by function. Genes and gene products governing steryl ester mobilization still await identification. Besides biochemical properties of enzymes involved in yeast neutral lipid synthesis and degradation, regulatory aspects of these pathways and cell biological consequences of neutral lipid depletion will be discussed in this minireview.

A large portion of cellular energy is required to accomplish biosynthetic reactions which utilize acetyl-CoA to form sterols and fatty acids. Storing these end products as steryl esters (STE) or triacylglycerol (TAG) facilitates the conservation of this chemical energy in a biologically inert form. TAG, however, is not only 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, but due to its low biological toxicity compared to free fatty acids it also provides an appropriate means of fatty acid "removal" from routes of complex lipid formation. Thus, on the one hand TAG is used as a fatty acid donor for membrane biogenesis as has been shown with yeast (Daum & Paltauf, 1980). On the other hand, it serves as a sink for highly abundant or unusual and thereby toxic fatty acids (Hunkova & Fencl, 1977; 1978) as well as for diacylglycerol (DAG), which is not only a precursor for TAG but also a key intermediate in phospholipid synthesis (see below) and at the same time a second messenger by binding to protein kinases (for reviews see: Goni & Alonso, 1999; Neri *et al.*, 2002). Similar properties may be attributed to STE whose formation is a critical homeostatic response to the formation of excess amounts of sterols and fatty acids in all eukaryotic cells. Thus, in addition to its storage function, STE biosynthesis is also a detoxification process which avoids possible membrane perturbation due to elevated levels of sterols, sterol precursors and free fatty acids. In fact, sterols have been known for a long time to play a critical role in establishing the appropriate fluidity of membranes (Lees *et al.*, 1979), the regulation of membrane-bound enzymes (Cobon & Haslam, 1973) and in maintaining permeability characteristics of membranes (Bard *et al.*, 1978; Kleinhans *et al.*, 1979). In summary, neutral lipid synthesis fulfils two important physiological roles, namely (i) storage of important lipid building blocks upon entry of cells into a resting (stationary) growth phase; and (ii) neutralization of excessive amounts of membrane-perturbing and signal-transducing molecules.

To date, four eukaryotic acyltransferase gene families have been identified which are involved in acyl-CoA-dependent or independent esterification reactions leading to neutral lipid synthesis (Oelkers & Sturley, 2004), namely acyl-CoA cholesteryl acyltransferase (ACAT), lecithin cholesteryl acyltransferase (LCAT), diacylglycerol acyltransferase 2 (DGAT2), and glycerol-3-phosphate acyltransferase and acyl-CoA lysophosphatidic acid acyltransferase (GPAT/AGAT) family. In the yeast *Saccharomyces cerevisiae* the enzymes which catalyze sterol and DAG esterification are members of three of these families: Dga1p belongs to the DGAT2 family, Lro1p to the LCAT family, and Are1p and Are2p are members of the ACAT family. Similar to other eukaryotes, yeast cells harbor more than one enzyme involved in the synthesis of TAG and STE. The involvement of overlapping biosynthetic systems for neutral lipid formation appears to be the result of differential regulation, alternative localization and different substrate specificities.

Neutral lipids are stored in the core of cytoplasmic lipid particles till hydrolysis directs return of their components to metabolic and/or catabolic pathways. The process of lipid depot formation is widely used in nature, and all types of eukaryotic cells contain intracellular lipid particles, also known as

lipid bodies, lipid droplets or, especially in plants, oil bodies, oleosomes or spherosomes (for reviews see: Zweytick *et al.*, 2000a; Brown, 2001). In multicellular organisms, STE and TAG are incorporated into lipoproteins which are secreted for transport to distant cells. All lipid storage compartments, however, share a general structure: a hydrophobic core of neutral lipids which is surrounded by a membrane monolayer of phospholipids containing a small amount of proteins. Lipid droplets from mammalian cells and plants harbor specific classes of polypeptides, namely perilipins (for reviews see: Londos *et al.*, 1995; Londos *et al.*, 1996; Londos *et al.*, 1999) and oleosins (for reviews see: Huang, 1992; 1996; Frandsen *et al.*, 2001), respectively, which are involved in formation, maintenance and mobilization of lipid depots. In contrast, yeast lipid particles contain a more complex set of enzymes which are mainly involved in lipid metabolism (Athenstaedt *et al.*, 1999b).

Neutral lipids stored in lipid particles can be mobilized by TAG lipases or STE hydrolases and thereby re-enter the pathways of cellular lipid metabolism. Despite their physiological importance, mechanisms of lipid mobilization from intracellular storage compartments are still insufficiently understood at the molecular and enzymatic levels. Exceptions are the two key enzymes of TAG mobilization in mammalian adipose tissue, the lipoprotein lipase (LPL) and the hormone-sensitive lipase (HSL). Whereas LPL mobilizes fatty acids from VLDL and chylomicron TAG in the capillary endothelium of adipose tissue (for reviews see: Mead *et al.*, 2002; Yin & Tsutsumi, 2003), HSL releases fatty acids from TAG stored in adipocyte cytosolic lipid droplets (for reviews see: Haemmerle *et al.*, 2003; Holm *et al.*, 2000; Kraemer & Shen, 2002; Yeaman, 2004). Enzymatic properties of HSL have been continuously and intensively studied for a long time. This enzyme which exhibits a broad specificity for lipid substrates such as TAG, DAG, cholesteryl esters and retinyl esters can also be found in several tissues different from adipose tissue. Surprisingly, little is known about TAG mobilization in mammalian liver cells, where HSL is not expressed (for a review see Gibbons *et al.*, 2000), and in plants (for reviews see: Huang, 1993; Mukherjee & Hills, 1994). Only most recently, TAG lipase Tgl3p localized to lipid particles of *S. cerevisiae* (Athenstaedt & Daum, 2003) was the first enzyme of this kind identified by function in yeast.

In this minireview we will summarize our present knowledge of formation, storage and mobilization of neutral lipids in yeast, especially in the budding yeast *S. cerevisiae*. Cell biological consequences of the loss of the complete neutral lipid synthesizing machinery will be described. Finally, the role of yeast lipid particles in neutral lipid storage and homeostasis will be discussed.

FORMATION OF NEUTRAL LIPIDS IN THE YEAST

Triacylglycerol biosynthesis

Synthesis of TAG in the yeast *S. cerevisiae* requires formation of its precursors phosphatidic acid and DAG. Two major *de novo* biosynthetic pathways which yield phosphatidic acid utilize either glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) as precursors (for a review see Athenstaedt & Daum, 1999). 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 phosphatidic acid. Alternatively, DHAP is acylated at the *sn-1* position by DHAP acyltransferase (DHAPAT). The product, 1-acyl-DHAP, is reduced by 1-acyl-DHAP reductase (ADR) to yield 1-acyl-G-3-P, which is further acylated to phosphatidic acid by AGAT. Phosphatidic acid can also be formed from phospholipids through the action of

phospholipases D, or by phosphorylation of DAG through DAG kinase. Activation of phosphatidic acid with CTP by a CDP-DAG synthase leads to the formation of CDP-DAG, the precursor for phosphatidylinositol, phosphatidylglycerol, cardiolipin, phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (for reviews see: Carman & Zeimetz, 1996; Daum *et al.*, 1998; Vance, 1998; Carman & Henry, 1999). Dephosphorylation of phosphatidic acid catalyzed by a phosphatidate phosphatase (PAP) yields DAG, which can also be formed from TAG by TAG lipases or from phospholipids through the action of phospholipases C. DAG is, on one hand, a precursor for aminoglycerophospholipid synthesis *via* the Kennedy pathway and therefore a key intermediate in membrane lipid formation (for reviews see: Kent, 1995; Carman & Zeimetz, 1996; Daum *et al.*, 1998; Carman & Henry, 1999; Voelker, 2000), and on the other hand, substrate to diacylglycerol acyltransferases (DAGATs) which convert DAG to TAG using different acyl donors.

In this review we will discuss only the last step in TAG synthesis, i.e. the conversion of DAG to TAG, in more detail. For a broader view of the preceding steps in TAG synthesis the readers are referred to most recent reviews (Sorger & Daum, 2003; Coleman & Lee, 2004).

Two different pathways lead to TAG formation in yeast

The terminal step in TAG synthesis is the esterification of DAG. In the yeast, conversion of DAG to TAG involves an acylation step utilizing either acyl-CoA or an acyl group of phospholipids as a source of fatty acids (Fig. 1). The enzyme representing the former group (Lehner & Kuksis, 1996) and at the same time the most prominent TAG-synthesizing enzyme is acyl-CoA diacylglycerol acyltransferase (DAGAT; EC 2.3.1.20). Enzymatic activity of DAGAT in yeast using endogenous 1,2-DAG as a substrate *in vitro* was localized to lipid particles a long time ago (Christiansen, 1978; 1979). Although ortho-

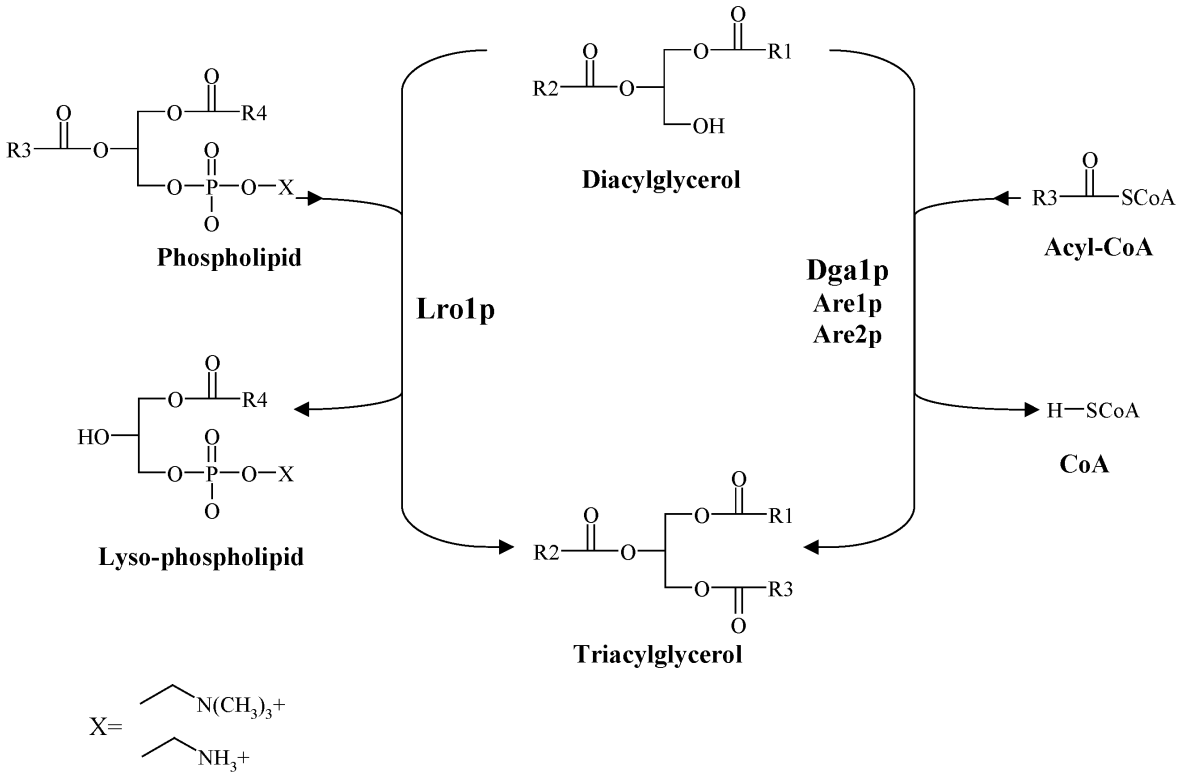

Figure 1. Triacylglycerol formation in yeast *via* different pathways

logs of the mammalian *DGAT1* encoding DAGATs homologous to acyl-CoA cholesterol acyltransferase (ACAT) were identified in humans (Oelkers *et al.*, 1998), *Ceanorhabditis elegans* (Bouvier-Nave *et al.*, 2000), mice (Cases *et al.*, 1998) and plants (Hobbs *et al.*, 1999; Routaboul *et al.*, 1999; Zou *et al.*, 1999), it took more than 20 years to characterize a corresponding yeast enzyme at the molecular level. Only recently, Lardizabal *et al.* (2001) reported the identification of two DAGATs from the oleaginous fungus *Mortierella ramanniana* unrelated to the ACAT family. Since homologues of these two polypeptides were identified in diverse organisms, the existence of another gene family, referred to as DGAT2, was proposed. Among the different homologues identified there was also a *S. cerevisiae* protein, which showed a 44% overall amino-acid sequence homology to the enzymes from *M. ramanniana*. Through heterologous expression in insect cells (Cases *et al.*, 2001; Lardizabal *et al.*, 2001) and analysis of the respective yeast deletion mutant (Oelkers *et al.*, 2002; Sandager *et al.*, 2002; Sorger & Daum, 2002) the yeast ORF *YOR245c* was shown to encode for a yeast DAGAT, in the following named Dga1p. Localization studies by Sorger and Daum (2002) using ^{14}C-labeled DAG and acyl-CoA as substrates unveiled a 70–90-fold enrichment of this DAGAT activity in lipid particles over the homogenate, but also a 2–3-fold enrichment in microsomal fractions. The activity of the enzyme was strongly dependent on the presence of K^+ and Mg^{2+}. Whereas the DAGAT activity in microsomes from a *dga1*Δ single mutant strain was only slightly decreased compared to wild type, the activity in lipid particles of this mutant was reduced to 5% of the wild type control. These data indicated that (i) Dga1p is the major or most likely the only acyl-CoA-dependent DAGAT in lipid particles, (ii) Dga1p is also localized to the endoplasmic reticulum (ER), and (iii) additional enzymes catalyzing acyl-CoA-dependent DAGAT activity must be present in the latter compartment. It has to be mentioned, however, that the subcellular localization of Dga1p is not completely agreed upon. Whereas Sorger and Daum (2002) located Dga1p-dependent DGAT activity to lipid particles and the ER, Oelkers *et al.* (2002) detected DGAT activity only in microsomes.

The enzyme responsible for the acylation of DAG using phospholipids as acyl donor had already been identified some years ago (Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2000). Sequence alignments with the human lecithin cholesterol acyltransferase (LCAT) unveiled *YNR008w* (*LRO1*; LCAT-related open reading frame) as the only yeast gene showing distinct sequence similarity with 27% overall identity and conservation of Ser181 and Asp345, two members of the LCAT catalytic triad. Lro1p, similar to LCAT, contains the serine lipase motif V.L(I/V)GHS.G at amino acids 318–326. Lro1p mediates esterification of DAG using the *sn-2* acyl group of phosphatidylcholine and phosphatidylethanolamine (Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2000) as acyl donor and thus exhibits phospholipid diacylglycerol acyltransferase (PDAT) activity. *In vitro* activity assays of the *lro1*Δ mutant and lipid analysis of yeast cells overexpressing *LRO1* confirmed the involvement of Lro1p in TAG formation. Acylation of DAG using acyl-CoA as substrate was unchanged in an *lro1*Δ mutant strain (Oelkers *et al.*, 2000; Sorger & Daum, 2002) indicating that Dga1p and Lro1p catalyze different reactions. Moreover, Lro1p activity was not detected in lipid particles as shown previously for Dga1p, but exclusively in the ER (Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2000).

Contributions of Dga1p and Lro1p to TAG synthesis

The existence of two different yeast enzymes involved in TAG synthesis raises the question as to the physiological relevance of this redundancy. Apart from different substrate specificities an unbalanced contribu-

tion of these two enzymes depending on the growth phase of a yeast culture was proposed. The quantitative contributions of Lro1p and Dga1p to TAG biosynthesis are still a matter of debate, though.

Substrate specificity of Lro1p and Dga1p

Substrate specificity of Lro1p was analyzed with respect to different acyl group positions, the head group of the acyl donor, the acyl group transferred and the acyl chains of the acceptor DAG molecule (Dahlqvist *et al.*, 2000). The experiments performed suggested that (i) the yeast PDAT preferentially catalyzes an acyl transfer from the *sn-2* position of a phospholipid to DAG; (ii) dioleoyl-phosphatidylethanolamine is used as acyl donor with four times higher efficiency than dioleoyl-phosphatidylcholine; (iii) a ricinoleoyl group at the *sn-2* position of phosphatidylcholine incorporates into TAG at a 2.5 times higher rate than an oleoyl group in the same position, whereas there is no preference for the transfer of vernoloyl groups over oleoyl groups; and (iv) DAG with a ricinoleoyl or a vernoloyl is a better acyl acceptor than dioleoyl-DAG. In summary, properties of substrates strongly influence the Lro1p-mediated acylation step. Interestingly, ergosterol is not a substrate for the yeast Lro1p although *LRO1* exhibits high homology to human LCAT.

In vitro assays identified oleoyl-CoA and palmitoyl-CoA as the preferred substrates of Dga1p, whereas myristoyl-CoA, stearoyl-CoA, arachidonyl-CoA and linoleoyl-CoA were used at a significantly lower rate (Oelkers *et al.*, 2002). Interestingly, strains disrupted in all genes involved in neutral lipid synthesis, except *DGA1,* had a fatty acid composition similar to wild type (Sandager *et al.*, 2002). Triple disrupted strains lacking *DGA1,* however, accumulated a significant amount of vaccenic acid, an unusual fatty acid found only at trace amounts in wild type. Moreover, the level of palmitoleic acid was elevated, whereas the amount of palmitic acid was reduced. Since the relative distribution of fatty acids was similar in TAG, STE and total lipids, the authors suggested a reduced TAG synthesis due to the lack of Dga1p to be responsible for the altered fatty acid composition.

Prevalence of either Dga1p or Lro1p depending on the growth phase

Deletion of *LRO1* or *DGA1* decreases TAG synthesis by up to 75% or 60% of wild type, respectively, the magnitude of the decrease depending on culture conditions (Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2000; Oelkers *et al.*, 2002; Sandager *et al.*, 2002; Sorger & Daum, 2002). Exponentially growing *dga1*Δ single mutants showed a less pronounced decrease in TAG amounts compared to cells grown to stationary phase (Oelkers *et al.*, 2002). Investigation of different stationary single disruptant strains, each lacking one of the four genes involved in neutral lipid synthesis (*DGA1*, *LRO1*, *ARE1* and *ARE2*, respectively), revealed *dga1*Δ as the sole single mutant with significantly reduced TAG levels (Sandager *et al.*, 2002). A major decrease in TAG synthesis in *lro1*Δ mutants was only observed for cells grown in the logarithmic growth phase (Oelkers *et al.*, 2000). Although the level of *LRO1* expression was found to be generally low, it was slightly higher during exponential growth compared to the stationary phase (Dahlqvist *et al.*, 2000).

In summary, Dga1p appears to catalyze the majority of cellular TAG synthesis in the stationary phase, and Lro1p is active mostly in the exponential phase. This view correlates (i) with the finding that expression of *LRO1* does not parallel TAG accumulation during late stationary phase (Dahlqvist *et al.*, 2000), and (ii) with transcriptional up-regulation of *DGA1* relative to *LRO1* during the diauxic shift and the stationary growth (Gasch *et al.*, 2000). In all growth phases, however, a 97% reduction of TAG synthesis in a *dga1*Δ*lro1*Δ double mutant was reported by Oelkers *et al.*

(2002), whereas Sorger and Daum (2002) postulated that *dga1*Δ*lro1*Δ cells grown to the late logarithmic growth phase still contained 20% of the wild type amount of TAG. These discrepancies, however, might be due to different wild type backgrounds used in these studies. Similar to *dga1*Δ and *lro1*Δ single mutants, the *dga1*Δ*lro1*Δ double mutant grows like wild type and forms lipid particles, although with a significant reduction in number and size (Oelkers *et al.*, 2002). These results indicate that Dga1p and Lro1p are the major contributors to DAG acylation in yeast, but alternative pathways for TAG synthesis exist as will be described below.

In addition to the different contributions of Dga1p and Lro1p to TAG synthesis depending on the growth phase, these two enzymes are affected by the availability of heme. Although *DGA1* has not been reported to be a hypoxic gene (Kwast *et al.*, 2002), results obtained by Ferreira *et al.* (2004) indicated that Dga1p is probably the major contributor to TAG synthesis in heme-deficient cells.

Alternative routes of DAG acylation

The mammalian *DGAT1* gene belongs to the acyl-CoA cholesterol acyltransferase (ACAT) gene family which includes the two mammalian *ACATs, ACAT1* and *ACAT2* (for reviews see: Buhman *et al.*, 2000; Buhman *et al.*, 2001; Cheng *et al.*, 2004) and the two yeast ACAT-related enzymes Are1p and Are2p which catalyze sterol esterification (Yang *et al.*, 1996; Yu *et al.*, 1996). This relationship led to the speculation that Are1p and Are2p may be responsible for residual TAG synthesis in a *dga1*Δ*lro1*Δ mutant. Sandager *et al.* (2002), using a set of triple deletion strains in which each strain expressed only one of the four genes *DGA1, LRO1, ARE1* or *ARE2*, discovered DAGAT activities of Are1p and Are2p, although at a very low rate. The finding that both ACAT related gene products in yeast harbor DAGAT activity contradicted previous reports postulating that only Are1p was involved in TAG synthesis whereas Are2p was restricted to sterol esterification (Sandager *et al.*, 2000). DAGAT activity of Are1p was also detected in our laboratory (D. Sorger, unpublished), whereas Oelkers *et al.* (2002) found that only Are2p mediated residual TAG formation in the absence of Dga1p and Lro1p. Thus, the contribution of Are-proteins to TAG formation is still controversial.

Another TAG forming activity different from Dga1p and Lro1p was detected in isolated lipid particles and microsomal fractions from *S. cerevisiae* (Sorger & Daum, 2002). *In vitro* experiments performed with radioactively labeled DAG and free fatty acids as substrates showed a moderate but significant TAG synthase activity, which occurred independently of acyl-CoA formation catalyzed by the fatty acid activator proteins Faa1p-Faa4p. Incorporation of free fatty acids into complex lipids of *S. cerevisiae* was shown previously (Wagner & Paltauf, 1994). Free fatty acids were preferentially incorporated into phospholipids *in vivo* suggesting a phospholipase A_2-dependent deacylation-reacylation mechanism. In addition, free fatty acids were incorporated into TAG, which gave raise to the speculation that a similar mechanism might be relevant for the formation of TAG. However, since a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ mutant was completely devoid of TAG (Sandager *et al.*, 2002), it was assumed that the observed acyl-CoA-independent TAG synthase activity was not relevant *in vivo*. Nevertheless, different pathways are involved in acylation of DAG in the yeast *S. cerevisiae* as summarized in Fig. 1.

In contrast to *S. cerevisiae*, where TAG formation appears to be distributed between lipid particles and microsomes, TAG biosynthesis in the oleaginous yeast *Rhodotorula glutinis* occurs only in part in the membrane fraction. Approximately 60% of total TAG synthase activity in this microorganism was found to be associated with a cytosolic 10S multienzyme complex which catalyzes TAG

biosynthesis during exponential growth (Gangar *et al.*, 2001a). This complex was isolated, purified and characterized and shown to contain lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, DAG acyltransferase, acyl-acyl carrier protein synthetase and acyl carrier protein. The complex is stabilized by protein–protein interactions and can incorporate acyl-CoA as well as free fatty acids into TAG and its biosynthetic intermediates. Free fatty acids are activated by a 35-kDa protein with acyl-acyl carrier protein (ACP) synthetase activity which catalyzes the ATP-dependent ligation of fatty acids with ACP to form acyl-ACP (Gangar *et al.*, 2001b). A dramatic reduction of the growth rate and TAG production was observed in the non-lethal mutants *tag1*Δ and *tag2*Δ bearing defects in the lysophosphatidic acid and the DAG acyltransferase activity, respectively (Gangar *et al.*, 2002). In the *tag1*Δ mutant, the amount of TAG was decreased by 90%, and also the formation of phosphatidic acid and DAG was reduced. In contrast, in the *tag2*Δ mutant only the TAG content was strongly decreased compared to wild type. The activity of the membrane-bound isoforms of TAG biosynthetic enzymes remained unaltered in both mutant strains.

Regulation of triacylglycerol synthesis

Although there is some evidence for growth phase and heme dependency of Dga1p and Lro1p, little is known about the regulation of TAG synthesis in yeast on its whole. One possible regulatory aspect might be a feedback regulation of TAG synthesis by sterol metabolism because defects in neutral lipid synthesis were observed in an *erg26-1* mutant (Baudry *et al.*, 2001). *ERG26* encodes a 4α-carboxysterol-C3 dehydrogenase, one of three enzymes required for the conversion of 4,4-dimethylzymosterol to zymosterol. Besides the expected changes in sterol and STE levels, temperature-sensitive *erg26-1* shifted to a non-permissive temperature showed a decreased rate of TAG biosynthesis, whereas DAG and monoacylglycerol (MAG) synthesis was increased. It remains to be elucidated whether accumulated zymosterol exerts its regulation effect at the transcriptional or post-translational level.

Steryl ester biosynthesis in yeast

It has been known for a long time that sterols can be esterified by two different types of enzymes. One of these enzymes is lecithin-cholesterol acyltransferase (LCAT), which catalyzes extracellular acyl-CoA-independent STE synthesis using phospholipids as acyl donors. The other enzyme is acyl-CoA-cholesterol acyltransferase (ACAT), which forms STE in an intracellular acyl-CoA-dependent reaction. Whereas the only member of the LCAT gene family in the yeast *S. cerevisiae*, Lro1p, is an intracellular protein that catalyzes DAG esterification (Dahlqvist *et al.*, 2000; Oelkers *et al.*, 2000), the two yeast representatives of the ACAT gene family, Are1p and Are2p, indeed catalyze sterol esterification.

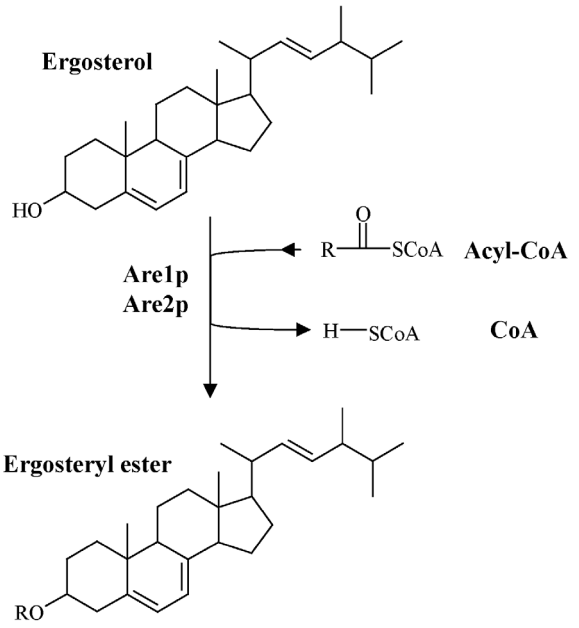

Figure 2. Steryl ester formation in yeast.

Instead of ergosterol precursor sterols can be utilized as substrates as well.

STE metabolism in yeast had been characterized extensively even before the respective genes were cloned. Early studies advocated a model of three metabolic sterol pools: a fixed amount of free sterol, a variable pool of bulk free sterol (Rodriguez *et al.*, 1985) and a STE fraction (Lewis *et al.*, 1987). The idea behind this hypothesis was that yeast cells must maintain an essential, low level of free sterol which is critical for growth. Additional amounts of sterol can form an expandable pool of this lipid class, but as soon as the amount of free sterols in the expandable pool exceeds a certain level, the rate of sterol esterification will increase. In contrast to the upper limitation for free sterols, the STE pool may range from virtually zero to a large percentage of total sterols (Taylor & Parks, 1978). The correlation between formation of STE and the growth stage of yeast is in line with this working model. Whereas the STE pool is very low during exponential growth when ergosterol is needed for membrane formation, it increases upon entry into the stationary growth phase due to the reduced requirement of ergosterol for membrane proliferation. *Vice versa*, when stationary cells are transferred into fresh medium, STE are rapidly hydrolyzed to free sterol and fatty acids which are utilized for the biogenesis of membranes (Taylor & Parks, 1978). In addition to depot formation, an additional "proof reading" function was suggested for the sterol esterification process by screening the types of sterols in the free sterol fraction and thereby ensuring that only ergosterol is available for new membrane synthesis (Taylor & Parks, 1981; Lewis *et al.*, 1987).

Differential contribution of Are1p and Are2p to sterol esterification

A rebound in the elucidation of STE metabolism was brought about by the landmark isolation of a human ACAT gene (Chang *et al.*, 1993) followed by the identification of the sterol *O*-acyltransferases (EC 2.3.1.26) Are1p and Are2p in *S. cerevisiae* (Yang *et al.*, 1996; Yu *et al.*, 1996). Are1p and Are2p exhibit 49% identity to each other and up to 20% homology to human ACAT which gave rise to their names as ACAT-related enzymes 1 and 2. Both enzymes are localized to the ER as demonstrated by enzymatic analysis (Zinser *et al.*, 1993) and microscopic visualization of GFP (green fluorescent protein) fusion proteins (Zweytick *et al.*, 2000b). In line with the evolutionary relationship mentioned above Yu *et al.* (1996) showed that human ACAT expressed in yeast *are1*Δ*are2*Δ double mutants catalyzed *in vitro* esterification of cholesterol and, to a lesser extent, ergosterol, although ergosterol oleate formation *in vivo* was restored only to about 8% of the original yeast reaction.

Deletion of both *ARE1* and *ARE2* completely abolishes sterol esterification, confirming the role of these two gene products as the only sterol esterifying enzymes. Unexpectedly and despite the major changes in sterol esterification conferred by the *are*Δ mutants, no obvious reduction of growth rates was observed (Yang *et al.*, 1996). Thorough analysis of growth of *are1*Δ*are2*Δ in competition with wild type, however, showed a decrease of the double mutant's growth rate to 65–70% after several rounds of transfer to fresh media (Zweytick *et al.*, 2000b). Examination of sporulating diploids revealed that a null mutation in *ARE2*, either alone or in combination with an *ARE1* null mutation, affected sporulation efficiency (Yu *et al.*, 1996). Furthermore, a large fraction of sporulating diploids arrested after the first meiotic division.

Different contributions of Are1p and Are2p to steryl ester synthesis

In contrast to a reduction of the STE level in an *are2*Δ single mutant to less than 26% of wild type, hardly any defect could be observed in an *are1*Δ mutant (Yang *et al.*, 1996; Yu *et al.*, 1996). Overexpression of Are1p or Are2p,

in an *are1*Δ*are2*Δ double mutant revealed that only Are2p could restore STE synthase activity to wild type level, whereas Are1p raised STE synthase activity only to values measured in *are2*Δ cells. Zweytick *et al.* (2000b) corroborated these findings by showing that Are2p activity in *are1*Δ microsomes was five times higher than Are1p activity in *are2*Δ microsomes. Taken together, these findings suggest that Are2p is the major STE synthase of the yeast. Additional support for the disproportional contribution of Are1p and Are2p to sterol esterification came from an investigation addressing the transcriptional regulation of the two enzymes (Jensen-Pergakes *et al.*, 2001). Using *ARE1* and *ARE2* promoter fusions to *lacZ* reporters it was demonstrated that transcriptional initiation through the *ARE1* promoter was significantly lower than through the *ARE2* promoter. Furthermore, the half-life of *ARE2* mRNA was approx. 12 times longer than that of the *ARE1* transcript. Thus, differences in Are1p- and Are2p-mediated sterol esterification activities under normal aerobic growth conditions are mainly results of the relative abundance of the specific transcripts.

Substrate specificities of Are1p and Are2p

Are1p and Are2p, however, do not only vary in their quantitative contribution to the sterol esterification process, but also in their substrate specificity. Analysis of the lipid pattern of single and double *are*Δ mutants by Zweytick *et al.* (2000b) revealed no preference for fatty acids of any of the Are-proteins, since the fatty acid patterns of STE from mutant strains were the same as from the wild type. The major fatty acid in STE was C16:1 followed by C18:1, whereas saturated fatty acids such as C14:0, C16:0 and C18:0 were incorporated only at minor amounts. The very long chain fatty acid C26:0 was detected only at 1% in STE. In contrast to the unchanged fatty acid composition, some significant differences were found between the sterol moieties of STE in the wild type, *are1*Δ and *are2*Δ. Generally, the major sterol esterified in wild type and *are1*Δ was ergosterol (50–60%), whereas precursor sterols accounted for 40–50% of total sterols esterified. A decrease of lanosterol and a slight increase of fecosterol was detected in STE of the *are1*Δ mutant. In *are2*Δ cells the portion of ergosterol esterified was decreased to 20% of total sterol whereas at the same time the levels of episterol and especially lanosterol in STE were significantly increased. Conclusively, Are2p seems to prefer ergosterol as a substrate compared to its precursors, whereas Are1p esterifies ergosterol and its precursors at nearly equal efficiency with a slight preference for lanosterol.

Studying the effects of overexpression of Are1p and Are2p in an *are1*Δ*are2*Δ double mutant background Jensen-Pergakes *et al.* (2001) confirmed the primary role of Are1p in esterifying sterol intermediates, especially lanosterol and, to a lesser extent, zymosterol. The ratio of esterified to free lanosterol was five-fold increased in a transformant overexpressing *ARE1* compared to *ARE2*. The ratio of esterified to free zymosterol in the *ARE1* overexpressant was only one-fourth relative to the strain overexpressing *ARE2*. The authors concluded from these results that the role of *ARE1* under aerobic growth conditions was to limit the conversion of lanosterol to zymosterol, thereby interrupting the sterol biosynthetic pathway in a way that allows storage of ergosterol precursors in lipid droplets as well as their liberation upon requirement. In contrast, the predominant role of Are2p was esterification of ergosterol. Concomitantly, it was shown that the *ARE1* promoter activity increased several-fold in strains like *erg2*Δ, *erg3*Δ and *erg6*Δ which accumulate ergosterol precursors.

Heme regulated expression of Are1p and Are2p

Another aspect addressing individual functions of Are1p and Are2p concerns the rela-

tionship between esterification of sterols and heme competency. Pioneering work with sterol and heme auxotrophic yeast strains showed a significant decrease in STE formation during supplementation with δ-aminolevulinic acid (ALA), the enzymatic product of Hem1p (Keesler *et al.*, 1992). However, since the uptake of exogenous sterol was inversely dependent on the concentration of δ-ALA, the decrease of STE formation was interpreted as direct response to the reduced amount of sterol available for esterification. Since δ-ALA did not affect the uptake of oleate, reduction of the esterification rate due to decreased amounts of oleate was ruled out. Interestingly, STE synthase activity itself was stimulated approximately four-fold in heme auxotrophic cells supplemented with 50 μg/ml of δ-ALA. This modulation of activity was found to be independent of the role of heme in the biosynthesis of sterols, unsaturated fatty acids and methionine. Consequently, heme competency was proposed to regulate both sterol uptake and STE synthase activity.

Although at that time neither *ARE1* nor *ARE2* had been identified, it was suggested, that two enzymes might be responsible for sterol esterification in yeast. This assumption was based on different dependencies on *HAP1*, a heme-responsive zinc finger transcription factor and redox sensing regulator of gene expression, and differential sensitivity against lovastatin, an inhibitor of HMG-CoA reductase, of δ-ALA-stimulated and basal STE synthase activities. Identification of Are1p and Are2p (Yang *et al.*, 1996; Yu *et al.*, 1996) allowed for further investigations on the influence of oxygen on these two enzymes. Anaerobic yeast cells supplemented with ergosterol and Tween 80 showed remarkably low STE levels irrespective of their growth phase. Labeling of TAG with [^{14}C]acetic acid, however, indicated that there was no general down regulation of storage lipid synthesis under these conditions (Valachovic *et al.*, 2001; Valachovic *et al.*, 2002). In contrast to aerobic grown cells, *are1*Δ cells cultivated anaerobically exhibited STE levels reduced to 25% of wild type, whereas no change was observed in *are2*Δ. It was hypothesized that different activities of Are1p and Are2p might reflect different roles of sterol esterification under (i) conditions optimal for ergosterol biosynthesis (storage of ergosterol in heme-competent aerobic cells) and (ii) suboptimal conditions promoting accumulation of ergosterol precursors and unnatural sterols from the environment (removal of detrimental sterols in heme-deficient or anaerobic cells). Preference of Are2p for ergosterol on the one hand and of Are1p for ergosterol precursors on the other hand might thus represent an adaptive advantage in aerobic or anaerobic cells, respectively.

In agreement with the results mentioned above and with a DNA microarray analysis of anaerobically growing cells, which revealed an eight-fold increased *ARE1* message level (ter Linde *et al.*, 1999), Jensen-Pergakes *et al.* (2001) supplied evidence for an opposite regulation of *ARE1* and *ARE2* by heme. Moreover, these authors showed that *ARE2* expression required the *HAP1* transcription factor, and both *ARE1* and *ARE2* were derepressed in a *rox1* (repressor of oxygen) mutant genetic background.

Independent of the effects described above it has to be mentioned that *ARE* gene expression is not subject to end product inhibition. Neither *ARE1* nor *ARE2*-driven expression of a lacZ reporter construct was altered in an *are*Δ background or in wild type overexpressing either *ARE1* or *ARE2* (Jensen-Pergakes *et al.*, 2001).

Sterol biosynthesis and sterol esterification are closely linked processes

The inability of yeast mutants to esterify sterols does not result in an effect on free sterol levels (Yang *et al.*, 1996). The fact, that the concentration of free sterols was roughly the same in the wild type and the different

*are*Δ single and double mutants was a first indication for a possible regulatory link between sterol biosynthesis and esterification. The sterol biosynthesis rate in the *are1*Δ*are2*Δ double mutant was one-half to one-third of that in wild type cells, although no changes were observed in the single *are*Δ mutants (Yang *et al.*, 1996). A similar feedback regulation of sterol biosynthesis by ACAT activity has also been observed in mammalian cells (Tabas *et al.*, 1986) and may be a common mechanism that maintains the intracellular amount of sterols at non-toxic concentrations. Indeed, the inhibition of ACAT in sterol-loaded cells induces cell death when extracellular sterol acceptors such as high density lipoproteins are absent (Warner *et al.*, 1995; Kellner-Weibel *et al.*, 1999).

An influence of STE synthesis on specific enzymes of the ergosterol biosynthetic pathway was shown with Erg3p (Arthington-Skaggs *et al.*, 1996) and Erg1p (D. Sorger *et al.*, in press). Expression of *ERG3* was down regulated three-fold in *are1*Δ*are2*Δ, but only 1.9-fold decreased in *are2*Δ and almost unchanged in *are1*Δ (Arthington-Skaggs *et al.*, 1996). The down regulation of the sterol biosynthesis in *are1*Δ*are2*Δ was paralleled by a 33% decrease of total sterol per cell dry mass. Furthermore, the ergosterol to sterol intermediates ratio in *are1*Δ*are2*Δ was significantly higher than in wild type or either single mutant. It was suggested that in the absence of sterol esterification all intermediates were accessible to sterol biosynthetic enzymes and thus ergosterol formation was not prevented.

In contrast to the regulation of *ERG3* expression, transcription of *ERG1* does not seem to be influenced by the presence of Are1p and Are2p (D. Sorger *et al.*, in press). Whereas Northern blot analysis revealed no difference in transcription levels of *ERG1*, the stability of Erg1p was strongly decreased in strains lacking Are1p and Are2p. Interestingly, this defect was more pronounced in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant lacking all four genes involved in neutral lipid synthesis than in *are1*Δ*are2*Δ.

Correlation between structure and function of Are-enzymes

Are2p was used as a model to define the relevance of conserved sequence domains for the activity of yeast ACAT-related enzymes (Guo *et al.*, 2001). On one hand, a regulatory role of an amino-terminal domain was recognized, and on the other hand two carboxy-terminal motifs were found to be required for catalytic activity. A serine-to-leucine mutation in the (H/Y)SF motif (residues 338–340), unique to sterol esterification enzymes, destroyed activity and stability of Are2p. Similarly, a tyrosine-to-alanine change in the FYxDWWN motif of Are2p (residues 523–529) yielded an enzyme with decreased activity and affinity for oleoyl-CoA. Mutagenesis of the tryptophan residues in the latter motif completely abolished activity. In human *ACAT1*, mutagenesis of the corresponding motifs (residues 268–270 and 403–409, respectively) also led to a loss of enzymatic activity. It was proposed that these polypeptide domains are required for sterol and acyl-CoA binding.

Sterol esterification as an essential process in lipid homeostasis

An interesting contribution to our knowledge of STE metabolism in yeast came from a screening for mutants inviable in the absence of sterol esterification (Tinkelenberg *et al.*, 2000). Mutations in *ARV1* (*ARE2* required for viability) rendered cells dependent on sterol esterification for growth, nystatin-sensitive, temperature-sensitive and inviable under anaerobic conditions. Deletion of *ARV1* also led to altered distribution and levels of intracellular sterol with an about 50% increase in free sterols and an about 75% increase in STE. Compartments affected by the elevation of the sterol level were the ER and

the vacuole, whereas in the plasma membrane fraction the sterol concentration decreased. Based on these data a role for Arv1p in sterol trafficking was proposed. Since *arv1*Δ*upc2-1* double mutants incorporated significantly less exogenous [^{14}C]cholesterol than *upc2-1* (a gain of function mutation that confers aerobic sterol uptake) cells, an additional role of Arv1p in sterol uptake was proposed. Interestingly, *ARV1* was found to be conserved in multicellular eukaryotes, and human *ARV1* was able to compensate for the yeast *arv1*Δ defect (Tinkelenberg *et al.*, 2000). Further studies showed that *arv1*Δ mutants also harbored a defect in sphingolipid metabolism (Swain *et al.*, 2002). These authors hypothesized that Arv1p may either primarily regulate sphingolipid metabolism and sterol defects observed result from improper sphingolipid homeostasis, or have a dual function regulating both sphingolipid and sterol metabolism and transport. A GFP-Arv1p hybrid protein was localized to the ER and the Golgi.

STORAGE OF NEUTRAL LIPIDS IN LIPID PARTICLES

Once synthesized yeast TAG and STE are deposited in organelles known as lipid particles. These particles are characterized by a rather simple structure consisting of a highly hydrophobic core of neutral lipids surrounded by a phospholipid monolayer with only a small amount of proteins embedded. The ratio between TAG and STE is approximately 1:1 (Schaffner & Matile, 1981). In *S. cerevisiae*, lipid droplets accumulate upon entry of the culture into the stationary phase and constitute up to 70% of the total lipid content of the cell (Clausen *et al.*, 1974; Leber *et al.*, 1994). Previously, lipid particles were considered only as a depot for lipids, but recent studies proposed a more complex role of these organelles in lipid biosynthesis, metabolism, degradation and trafficking. In fact, all lipid particle proteins identified so far by function are enzymes of lipid metabolic pathways. Interestingly, these proteins are either dually located in lipid particles and the ER, or they contribute to metabolic sequences mainly occurring in the ER. One example for the redundancy of enzymes in these two compartments concerns the biosynthesis of TAG. The ER contains the whole set of TAG-synthesizing enzymes and is the major site of TAG formation. However, lipid particles are able to form TAG autonomously: In the first part of this pathway, Gat1p, Ayr1p and Slc1p, present at significant amounts in lipid particles (Athenstaedt & Daum, 1997; Athenstaedt *et al.*, 1999a; Athenstaedt & Daum, 2000), catalyze phosphatidic acid formation. Then, phosphatidate phosphatase mainly localized to the cytosol but with access to the surface of lipid particles, converts phosphatidic acid into DAG. Finally, the lipid particle proteins Faa1p and Faa4p (acyl-CoA forming fatty acid activators) (Athenstaedt *et al.*, 1999b) and the TAG synthase Dga1p (Sorger & Daum, 2002) complete the ER-independent TAG synthesis.

An example for the distribution of a pathway between ER and lipid particles is the biosynthesis of ergosterol. Whereas the majority of enzymes of ergosterol synthesis are exclusively associated with the ER (Daum *et al.*, 1998), three proteins, namely Erg1p, Erg6p and Erg7p, are either localized solely to lipid particles or are distributed between the ER and lipid particles (Zinser *et al.*, 1993; Leber *et al.*, 1998; Milla *et al.*, 2002). The question as to the physiological relevance of the sterol biosynthetic pathway being distributed between two subcellular fractions remains open. The fact that three key enzymes of yeast sterol biosynthesis are subject to this distributive phenomenon suggests that the presence of enzymes of a pathway in two different organelles may provide an additional regulatory possibility at the organelle level, which has not yet received sufficient attention. In the case of Erg1p, the subcellular distribution appears to be even more important,

because the portion of this protein present in the lipid particle fraction is enzymatically inactive, whereas Erg1p of the ER is active (Leber *et al.*, 1998). The presence of sterol-synthesizing enzymes in two organelles also raises the question of interorganelle migration of sterol intermediates. Similar to sterols, early intermediates of phospholipid biosynthesis are also shuffled between lipid particles and the ER (Athenstaedt & Daum, 1999). Surface contact of the two organelles or even specific proteins may play a role in this process. Thus, interplay of lipid particles and ER may affect the regulation of certain lipid biosynthetic pathways.

Another role suggested for lipid particles is their participation in the transport of ergosterol to the plasma membrane. This view was supported by recent findings in our laboratory (D. Sorger *et al.*, in press) which showed a discrepancy between ergosterol levels in total membranes and the plasma membrane from neutral lipid deficient strains. Whereas the amount of free ergosterol in the plasma membrane from the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant was only 60%, and in an *are1*Δ*are2*Δ double mutant 80% of wild type level, no such decrease was observed in bulk membranes of these strains. Thus, lipid particles of the yeast may play a regulatory role in sterol/STE homeostasis through an esterification/hydrolysis mechanism.

The interaction with the ER also appears to be important for the biogenesis of lipid particles. A widely accepted model suggests that newly synthesized TAG and STE accumulate in the hydrophobic region of the ER phospholipid bilayer membrane. Membrane regions containing proteins of lipid metabolism which lack transmembrane domains may preferentially be incorporated into nascent lipid particles. Eventually, a droplet of neutral lipids surrounded by a phospholipid monolayer containing certain proteins disconnects and is released into the cytosol (Fig. 3). This model is strongly supported by the accumulation of TAG within the ER of developing plant seeds (Lacey *et al.*, 1999). However, distinct signals which direct ER-derived proteins to the newly formed monolayer of a budding lipid particle have not yet been identified. As already mentioned above, poly-

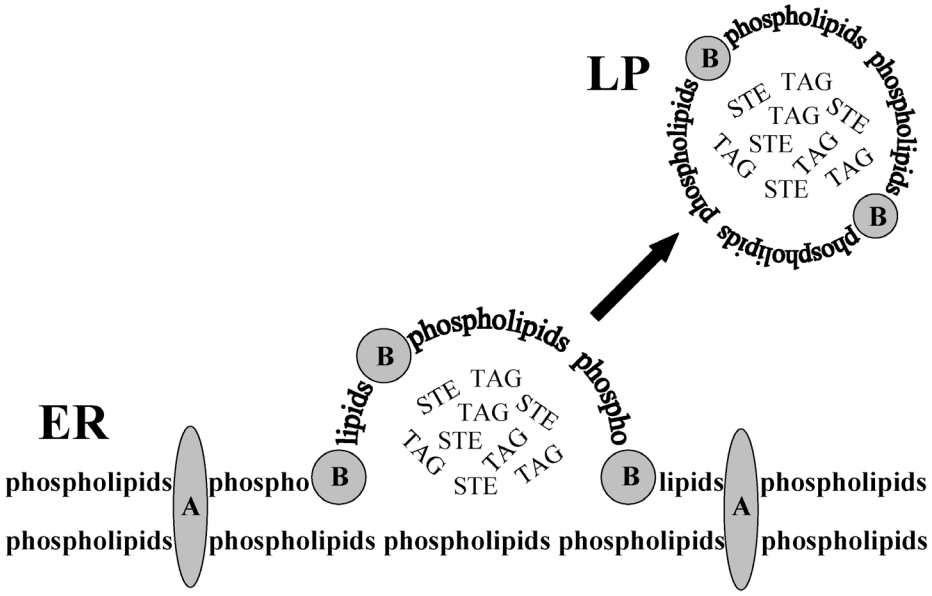

Figure 3. Model of lipid particle biogenesis.

A, transmembrane protein; B, protein without transmembrane spanning domain; ER, endoplasmic reticulum; LP, lipid particle; TAG, triacylglycerol; STE, steryl esters.

peptides lacking transmembrane domains are candidate lipid particle proteins. In addition, C-terminal hydrophobic domains seem to play a crucial role in the partitioning of proteins between the ER and lipid particles. Most recently, we (Müllner *et al.*, 2004) showed that three enzymes of the ergosterol biosynthetic pathway, namely Erg1p, Erg6p and Erg7p, all of them being lipid particle proteins in wild type cells, lost their ability to associate with lipid particles completely or at least in part when their hydrophobic C-termini were removed. The fact that these truncated proteins were not randomly mislocalized within the cell but preferentially retained in the ER confirmed the hypothesis of lipid particles originating from the ER. This view was supported recently in our laboratory (D. Sorger *et al.*, in press) by localization studies of Erg1p, Erg6p, Erg7p and Ayr1p (lipid particle proteins in wild type) in the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant which lacks lipid particles. Loss of their natural environment caused retaining of these lipid particle proteins in the microsomal fraction.

Through the studies of Sandager *et al.* (2002) it became evident that functionality of the neutral lipid synthesizing enzymes Lro1p, Dga1p, Are1p and Are2p is essential for the formation of lipid particles. The question remained, however, to what extent each protein contributes to lipid particle proliferation. Microscopic inspection of *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ harboring plasmid-born inducible TAG- and STE-synthesizing enzymes shed some light on this aspect (D. Sorger *et al.*, in press). Using the fluorescent dye Nile Red it was shown that already after 15 min of induction strains expressing Dga1p or Lro1p formed lipid particles. In contrast, it took 60 min of induction in the Are2p expressing transformant for lipid particles to become visible. Thus, it was assumed that TAG synthesis contributes more efficiently and rapidly to lipid particle biogenesis than synthesis of STE.

MOBILIZATION OF NEUTRAL LIPIDS

Utilization of TAG and STE from lipid particles requires the action of TAG lipase(s) (EC 3.1.1.3) and STE hydrolase(s) (EC 3.1.1.13). Some proteins associated with the phospholipid monolayer of lipid particles, such as mammalian perilipins and oleosins of plants, were assumed to be involved in the mobilization of the neutral lipid core of the particle by serving as docking and/or activating proteins for TAG lipases and STE hydrolases. Alternatively, perilipins and oleosins were assumed to protect the stored lipids from random degradation (Huang, 1992; Londos *et al.*, 1995; Murphy & Vance, 1999; Brasaemle *et al.*, 2000; Sztalryd *et al.*, 2003). Analysis of the most abundant yeast lipid particle proteins by mass spectrometry (Athenstaedt *et al.*, 1999b) led to the identification of several polypeptides with unknown function. None of these polypeptides, however, was homologous to perilipins and oleosins. Nevertheless, it was tempting to speculate that at least some of these novel lipid particle proteins may also play a role in neutral lipid mobilization.

Triacylglycerol lipases

TAG lipases have been one of the few white spots in lipid metabolism of the yeast *S. cerevisiae* for a long time. Early work in the 70ies (Schousboe, 1976a; 1976b) described a TAG lipase activity in the yeast mitochondrial fraction, but the polypeptide catalyzing this reaction was not identified. In the course of the yeast genome sequencing project, several putative yeast lipases were detected by homology to lipolytic enzymes from other organisms. Three polypeptides encoded by the ORFs *YDL109c*, *YGL144c* and *YDR444w* contain lipase-active site motifs (www.proteome.com/), and a mutant deleted of *YDL109c* exhibited an increased TAG level (Daum *et al.*, 1999). None of these ORFs, however, was

shown to encode a TAG lipase by enzymatic analysis of the gene product. Besides these polypeptides, two proteins of unknown function, named Tgl1p and Tgl2p, were postulated to be yeast TAG lipases based on their homology to rat, human and *Pseudomonas* lipases (Abraham *et al.*, 1992; van Heusden *et al.*, 1998). Recently, Cvt17p, which contains a motif conserved in esterases and lipases (Teter *et al.*, 2001), was also proposed to be a lipase involved in degradation of autophagic vesicles in the vacuole. TAG lipase activity, however, was neither demonstrated for Tgl1p nor for Tgl2p and Cvt17p by function.

Computational analysis of yeast lipid particle proteins revealed that some of these polypeptides contain a lipase motif, namely gene products of *TGL1*, *YOR059c* and *YJU3*. TAG levels in *tgl1*Δ, *yor059c*Δ and *yju3*Δ were similar to wild type, but another deletion mutant, *ymr313c*Δ, lacking a gene product without homology to known lipases showed increased levels of TAG (Athenstaedt *et al.*, 1999b). Indeed, the gene product of *YMR313c*, in the following named Tgl3p, was shown (i) to be involved in TAG catabolism *in vivo*; (ii) to exhibit TAG lipase activity in highly purified lipid particles; and (iii) to be enzymatically active *in vitro* when purified close to homogeneity (Athenstaedt & Daum, 2003). Although Tgl3p does not exhibit overall homology to other known lipases, the consensus sequence GXSXG present in Tgl3p is in line with its identification as a lipolytic enzyme. Most recently, a lipase from *Candida parapsilosis* was identified, which also contains this consensus motif and lacks similarity with other lipases (Neugnot *et al.*, 2002). The question remained whether or not Tgl3p is the only TAG lipase in *S. cerevisiae*. Incomplete mobilization of TAG in a *tgl3*Δ mutant treated with cerulenin, an inhibitor of fatty acid synthesis, rather suggested the existence of additional yeast TAG lipases, probably with minor enzymatic activities (Athenstaedt & Daum, 2003).

Localization of Tgl3p in lipid particles raises the question as to the coordinate formation, storage and mobilization/degradation of TAG. How can Tgl3p be prevented from permanently degrading TAG stored in lipid particles? A mechanism of lipolysis/re-esterification as reported for other experimental systems (Wiggins & Gibbons, 1992; Yang *et al.*, 1995) may also be anticipated for yeast. Regulation *in vivo* through presently unknown mechanisms might favor one or the other direction of this enzymatic step leading either to formation or mobilization of TAG depots.

Steryl ester hydrolases

Little is known about STE hydrolases in yeast. Taketani *et al.* (1978) detected the highest specific activity of STE hydrolase of *S. cerevisiae* in mitochondria. This finding, however, was scrutinized by Zinser *et al.* (1993), who reported localization of the highest enzyme activity in the plasma membrane and in secretory vesicles. This discrepancy may be due to different cell fractionation protocols and the use of different yeast strains.

Pathway(s) and mechanism(s) of STE mobilization in yeast are poorly characterized. Ongoing membrane proliferation appears to be a driving force for the release of sterols from STE of lipid particles, a process which is not mediated by microtubuli-dependent vesicle flux (Leber *et al.*, 1995). In analogy to Tgl3p of lipid particles which hydrolyzes TAG it was speculated that a STE hydrolase may also be associated with the lipid particle surface. Among the major lipid particle proteins of *S. cerevisiae* (Athenstaedt *et al.*, 1999b) two gene products turned out STE hydrolase candidates. Strains deleted of *YKL140w* (*TGL1*) and *YBR177c* contain higher amounts of STE than wild type. Functional analysis, however, identified the *YBR177c* gene product as an alcohol acyltransferase which forms ethylhexanoate from ethanol and hexanoyl-CoA

(Malcorps & Dufour, 1992; Mason & Dufour, 2000). The question as to the cellular function of *TGL1* is still open although significant sequence similarity was found between Tgl1p and TAG lipases from rat salivary gland and human gastric tissue (Abraham *et al.*, 1992). Tgl1p was described to enhance steroid productivity, probably through both the availability and/or trafficking of the CYP11A1 (cytochrome P450 side chain cleavage enzyme) substrate (Duport *et al.*, 2003). The idea of Tgl1p being a STE hydrolase was supported by an investigation dealing with lipid dynamics in yeast under heme-induced starvation of unsaturated fatty acids and/or sterols (Ferreira *et al.*, 2004). Deletion of *TGL1* in a *hem1*Δ mutant background resulted in an increased STE level which is in agreement with results reported earlier by Athenstaedt *et al.* (1999b). A closer examination of this strain revealed a specific enrichment of ergosterol and of unsaturated fatty acids in the STE fraction. Heme-depleted *tgl1*Δ*hem1*Δ double mutants showed an about 2-fold decrease of free ergosterol compared to the corresponding *hem1*Δ single mutant. A possible involvement of Tgl1p in the specific hydrolysis of ergosteryl esters and STE containing unsaturated fatty acid is consistent with the finding that *TGL1* is up-regulated under conditions of unsaturated fatty acid and ergosterol depletion, e.g. under oxygen limitation (Kwast *et al.*, 2002). Therefore, sterol homeostasis under heme depletion may be the result of a complex balance between storage of specific toxic intermediates in STE, especially through catalysis of Are1p, and the release of ergosterol from ergosteryl esters by Tgl1p to warrant at least transient membrane proliferation and residual growth. Nevertheless, the finding that upon heme-induced lipid starvation STE pools were completely emptied even in the absence of Tgl1p (Ferreira *et al.*, 2004) ruled out a role of this protein as a major STE hydrolase.

CONSEQUENCES OF NEUTRAL LIPID DEPLETION

Neutral lipids are not a wasteful resource and genes involved in their homeostasis are certainly not neutral in terms of evolutionary selection. Surprisingly, a yeast mutant strain lacking all four genes involved in neutral lipid biosynthesis, *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ, grows like wild type under standard conditions. Substantiating the role of Are1p, Are2p, Dga1p and Lro1p as the only proteins catalyzing the final steps in neutral lipid synthesis, this strain is devoid of storage lipids and consequently of lipid particles (Sandager *et al.*, 2002). In general, only minor differences between the quadruple disrupted strain and the wild type regarding the total lipid composition were observed in the exponential phase, where the amount of storage lipids in the wild type is low. In the stationary phase, when wild type cells accumulate neutral lipids, the quadruple mutant showed a 2.5-fold increase in fatty acids and a 3.7-fold decrease in DAG.

Even though it is surprising that *S. cerevisiae* strains deficient in neutral lipid synthesis exhibit normal growth kinetics, it has to be considered that under stress-free conditions (laboratory conditions) in the presence of sufficient nutrients neither storage of lipid resources nor detoxification by removal of membrane-perturbing components (excess of sterols or fatty acids) appears to be essential for life. However, when the energy supply is limited or cells are physiologically stressed, neutral lipid storage is likely to become more relevant. In line with this hypothesis the quadruple mutant showed reduced viability under nitrogen starvation (Sandager *et al.*, 2002).

Recent work in yeasts different from *S. cerevisiae* shed new light on the relevance of neutral lipid synthesis. Decreased TAG formation either by lowering the nutrient level or by deleting the enzymes of the soluble TAG

biosynthetic pathway was accompanied by a significant growth reduction of the oleaginous yeast *Rhodotorula glutinis* (Gangar *et al.*, 2002). An even more surprising result was presented in the course of the identification of the two enzymes responsible for TAG synthesis in the yeast *Schizosaccharomyces pombe*, Dga1p and Plh1p (*P*ombe *L*RO1 homolog 1) (Zhang *et al.*, 2003). The double deletion mutant *dga1*Δ*plh1*Δ of *Schizosaccharomyces pombe*, unable to synthesize TAG, completely lost viability upon entry into the stationary phase. This loss of viability was accompanied by the appearance of prominent apoptotic markers such as nuclear DNA fragmentation, exposure of phosphatidylserine to the cell surface and generation of reactive oxygen species. Deletion of Dga1p and Plh1p resulted in a highly elevated level of DAG (in contrast to the reduced level of DAG in *S. cerevisiae*) which was proposed to mediate lipoapoptosis in this yeast. Of course, it has to be considered that the fission yeast *Schizosaccharomyces pombe* and the budding yeast *S. cerevisiae* are different microorganisms. Nevertheless, the different response of these two cell types to the loss of neutral lipid synthesis is puzzling.

CONCLUSIONS AND FUTURE PERSPECTIVES

Although during the last few years the full set of genes for neutral lipid synthesis in the yeast *S. cerevisiae* has been identified, several important questions remained unanswered. The presence of enzymes with overlapping function in the esterification of sterols and the conversion of DAG to TAG raises the question as to their physiological relevance. Further investigations will be needed to elucidate the effects of various environmental influences on the contribution of Are1p and Are2p on the one hand and of Dga1p and Lro1p on the other hand to neutral lipid formation. However, explaining the redundancy of enzymatic systems will not be the only challenge for future research. To find out the reasons for the distribution of pathways forming the same end product among different organelles will be another important question

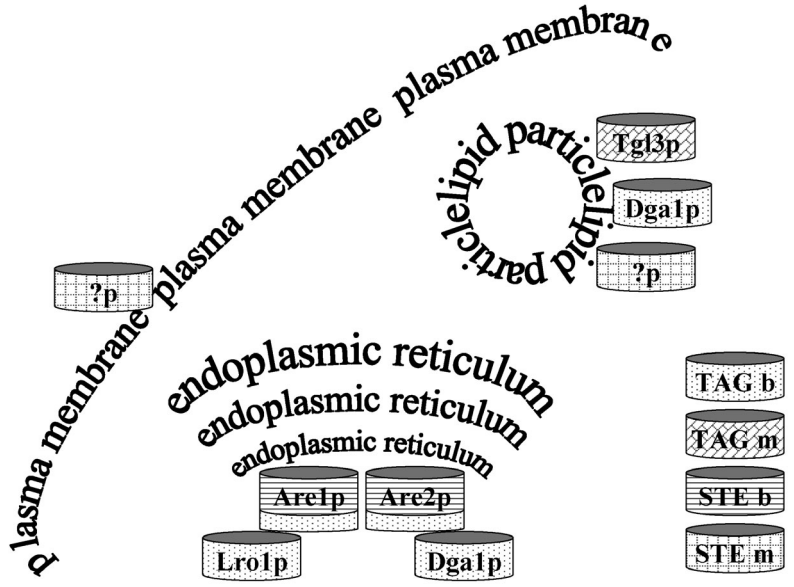

Figure 4. Interplay of endoplasmic reticulum, lipid particles and plasma membrane in neutral lipid homeostasis in the yeast *Saccharomyces cerevisiae*.

TAG b, protein involved in triacylglycerol biosynthesis; TAG m, protein involved in triacylglycerol mobilization; STE b, protein involved in steryl ester biosynthesis; STE m, protein involved in steryl ester mobilization; ?p, unknown protein(s)

for cell biologists. A further aspect of particular interest related to neutral lipid synthesis is the biogenesis of lipid particles, an organelle which attracted more and more attention during the last decade. Regulatory aspects of STE and TAG synthesis and the related formation of neutral lipid depots in the form of lipid particles are far from being clear. Finally, routes and mechanisms relevant for mobilization and utilization of yeast neutral lipids need to be investigated in more detail. Although many of the players in the neutral lipid game have been identified by function, a number of proteins involved have still to be characterized at the molecular level. Future research will most likely also be focused on the understanding of the active role of lipid particles in lipid homeostasis. Since steps of neutral lipid metabolism seem to be distributed between lipid particles, the ER and probably the plasma membrane (Fig. 4), special emphasis will be given to the investigation of the interplay of these subcellular compartments.

The availability of the whole yeast genome sequence in combination with the ease of genetic manipulation of this microorganism renders *S. cerevisiae* an extraordinarily powerful experimental system to address all these questions. It is remarkable that basic biological structures and processes involved in neutral lipid metabolism have been conserved throughout the development of eukaryotes. In fact, all mammalian enzymes involved in TAG and STE formation, storage and mobilization identified so far have a yeast counterpart. Consequently, information obtained with the unicellular eukaryote yeast provides a useful contribution to our general understanding of lipid metabolism in multicellular eukaryotes as well.

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