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Lipid storage and mobilization in the yeast *Saccharomyces cerevisiae*

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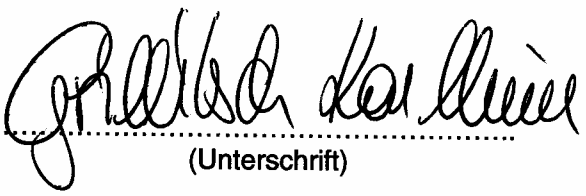
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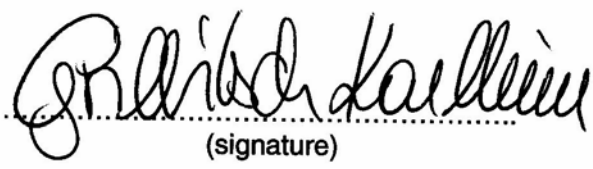
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Zusammenfassung

In der Hefe *Saccharomyces cerevisiae* gibt es so wie in anderen Eukaryonten und manchen Prokaryonten Zellorganellen, die nur durch eine Monolayer-Membran aus Phospholipiden geschützt werden, die sog. Lipid Partikel (LP). Dieser Monolayer grenzt den hydrophoben Kern ab, der aus den Neutrallipiden Sterolestern (STE) und Triacylglyceriden (TAG) besteht. In der Phospholipidmembran sind einige wenige spezifische Proteine enthalten. In dieser Dissertation wurden Depotbildung und Mobilisierung der Neutrallipide der Hefe untersucht. Als erstes wurden die biochemischen Eigenschaften der drei STE-Hydrolasen Tgl1p, Yeh1p und Yeh2p studiert, die gewisse Substratspezifitäten aufweisen. Die drei Enzyme sind nicht auf Bildung eines Produkts limitiert, tragen jedoch zur Sterol-Homöostase in Hefe bei. Dieses Gleichgewicht ist auch mit Zellpolarität verknüpft. Es konnte gezeigt werden, dass zwei Effektoren der Zellpolarität, Ste20p und Cla4p, als Negativ-Modulatoren der Sterolbiosynthese fungieren und dadurch indirekt die Zellpolarität beeinflussen. Ein Schwerpunkt dieser Arbeit war dem molekularen Aufbau der LP gewidmet, vor allem unter dem Einfluss unterschiedlicher Wachstumsbedingungen. Bei einem Wechsel von Glucose zu Ölsäure als C-Quelle wurde diese einfach ungesättigte Fettsäure präferenziell in TAG und die meisten Phospholipide eingebaut. Besonders bemerkenswert war die Verschiebung des 1:1 Verhältnisses von TAG zu STE bei Glucose als C-Quelle zu wesentlich höheren Werten mit Ölsäure. Der Wechsel der C-Quelle bewirkte auch eine Veränderung im Proteinmuster der LP. Dies konnte in einer kombinierten Lipidom/Protein-Analyse gezeigt werden, die auch zur Identifizierung einiger neue LP Proteine führte. Schließlich wurden Probleme des Targetings und der Topologie von LP am Beispiel der Squalenepoxidase Erg1p untersucht. Der Hauptteil des Proteins ist dem Cytosol zugewandt und nur ein kleiner Teil von der Membran geschützt. Diese Anordnung scheint typisch für LP Proteine zu sein.

Summary

The yeast *Saccharomyces cerevisiae* contains like higher eukaryotic cells (mammals and plants) and Gram-positive bacteria a specified organelle for lipid storage, the lipid particle (LP). Unlike other organelles LP are covered by a phospholipid monolayer that protects its hydrophobic interior formed from densly packed non-polar lipids steryl esters (STE) and triacylglycerols (TAG). Moreover, LP contain a small but specific set of proteins. In this Thesis, storage and mobilization of yeast neutral lipids was studied. First, biochemical properties of the three STE hydolases, Tgl1p, Yeh1p and Yeh2p were investigated. Analysis of enzymatic properties revealed distinct substrate specificities of the three proteins and involvement in sterol homeostasis. Sterol homeostasis is also linked to cell polarity. We showed that two effectors of cell polarity, Ste20p and Cla4p, function as negative modulators of sterol biosynthesis. A major part of this Thesis was devoted to description of the molecular composition of the yeast LP and its modulation upon changes in cultivation conditions. For this purpose, LP from cells grown on either glucose or oleate were analyzed. Strong incorporation of the mono-unsaturated oleic acid into TAG and most phospholipids was observed upon shifting cells to oleate medium. Most notably, the balanced 1:1 ratio of TAG to STE in cells grown on glucose was strongly increased on oleate. Change of the medium also led to changes in the LP protein pattern. This was demonstrated by a combined lipidomic/proteomic approach which also revealed several novel putative LP proteins. Finally, LP protein targeting and topology were studied using the squalene epoxidase Erg1p, a typical LP protein, as a model. These studies showed that the majority of the protein faced the cytosol, and only a small part was protected by the membrane. We assume that this may be a general feature of LP proteins.

General Introduction

The yeast *Saccharomyces cerevisiae* contains four enzmes that are capable of producing non-polar lipids. The two yeast steryl ester (STE) synthases (Are1p, Are2p) and the two triacylglycerol (TAG) synthases (Dga1p, Lro1p) are localized to the endoplasmic reticulum (ER). This compartment is the origin of a lipid storage organelle which is called lipid particle (LP) or lipid droplet. The four acyltransferases Are1p, Are2p, Dga1p and Lro1p appear to initiate the formation of LP by synthesizing its major constituents, TAG and STE. After segregation of the newly produced neutral lipids into concentrated pools between the two leaflets of the ER, a small droplet of high fat content is formed. With proceeding time the droplet gets enlarged, and once its size exceeds accommodation of the ER a nascent LP may bud off the membrane by a mechanism which is not yet understood. Dga1p is the only neutral lipid synthesizing enzyme that is dually located, namely in the ER and in LP. A phospholipid monolayer which forms the surface of LP is also assumed to be result of the budding process from the ER. This monolayer protects the hydrophobic core of the LP from its hydophilic enviroment with the polar headgroups facing the cytosol and the hydrophobic tails oriented towards the hydrophobic core of the LP (Czabany *et al.*, 2007). A detailed description of the major compounds of the LP was one of the major objectives of this Thesis. Analysis of the LP was not restricted to normal cultivation conditions when glucose is used as a carbon source, but was extended to the possible influence of alternative carbon sources, e.g. oleate, on the molecular equipment and assembly and LP.

The precence of a small but specific set of proteins that are embedded in the phospholipid monolayer is a further characteristic of LP. Many LP proteins are involved in biological processes directly or indirectly linked in lipid metabolism (Athenstaedt *et al.*, 1999). So far, no signalling sequence or other sequence specificities could be attributed to this class of

proteins which would help to explain protein targeting to LP. The presence of several hydrophobic stretches within LP proteins is so far the only hint how these proteins might get attached to the phospholipid monolayer (Müllner *et al.*, 2004). Neutral lipid storage compartments exist in all types of eukaryotic cells (mammalian lipid droplets or plant oil bodies) as well as in some prokaryotic cells. In the case of LP proteins from mammalian lipid droplets and plant oil bodies more conclusive data on sequence specifications for LP localization were presented (Zehmer *et al.*, 2008). In these types of cells, highly specific proteins such as the perilipins of oleosins were detected. In the yeast, homologues of these proteins have not been identified so far. A major aim of this Thesis was an improved proteome analysis of yeast LP. Along that way one characteristic LP resident protein (Erg1p) was used for topology studies that aimed to elucidate mechanism(s) governing targeting and assembly of proteins ending in this organelle.

The importance of LP in storage of fatty acids and sterols in the biological inert form of STE and TAG is well accepted. When cells find themselves in the need of nutritional depletion, stored lipids can be mobilized to provide building blocks for bulk membrane biosynthesis and to supply substrates for energy production. Yeast enzymes which hydrolyze stored STE are Tgl1p, Yeh1p and Yeh2p (Zinser *et al.*, 1993; Köffel *et al.*, 2005). Characterization of these enzymes which contribute to the mobilization of STE from LP was another aim of this work. Two of these enzymes, namely Tgl1p and Yeh1p are LP components. This evidence is in line with previous findings that LP are not only storage organelles, but also contribute actively to metabolic processes. Moreover, stabilizing property can be attributed to LP, e.g. for proteins such Erg1p (Sorger *et al.*, 2004). Finally, however, LP are thought to be important for caging hydrophobic molecules which are potentially toxic (fatty acids, squalene) when accumulating within the cell (Milla *et al.*, 2002).

Another aspect related to the role of LP is sterol homeostasis. Sterols are distributed heterogenously throughout the eukaryotic cell. Ergosterol is one of the most important lipid components of yeast membranes. Biosynthesis of ergosterol is catalyzed by a set of enzymes which can be assigned to the *ERG* gene family with many of these proteins being located in the ER, but some in LP. Disturbance of sterol homeostasis may be harmful for the cell as any alteration may negatively influence physical properties of cellular membranes. Membrane proteins might as well rely on a certain threshold of sterols in their environment to maintain proper function(s) (Schulz and Prinz, 2007). Sterols, however, are not only important structural membrane compounds but also seem to play a crucial role in cell polarization (Bagnat and Simons, 2002; Jin *et al.*, 2008). As examples, Ste20p as well as Cla4p, both members of the p21-activated kinase (PAK) family, are Cdc42p effectors that regulate cell polarization. Tiedje C. *et al.* (Tiedje *et al.*, 2007) showed recently that Ste20p, a member of the PAK family, binds to enzymes involved in sterol biosynthesis. A possible regulating function of the two Cdc42p effectors mentioned above on sterol biosynthesis during polarization was a further objective of investigation for this Thesis.

In summary, the following chapters of this Thesis will describe various aspects and facets of lipid storage in the yeast. Lipid accumulation may be regarded as good or bad. In the case of plants, fat storage is important for the production of nutrients. In contast, excess of fat in a human body may lead to severe diseases. The molecular understanding of neutral lipid storage and mobilization is therefore important for balanced cell growth and function not only in *Sacccharomyces cerevisiae*, our model cell, but also with higher eukaryotes. Thus, our results may become relevant in the future for a better understanding of lipid storage in medical science and biotechnology.

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Chapter 1

Non-polar lipid synthesis and turnover in yeast

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Abbreviations: 1-acyl DHAP, 1-acyl dihydroxyacetone phosphate; 1-acyl G-3-P, 1-acyl glycerol-3-phosphate; ACAT, acyl-CoA:cholesterol acyltransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGPP, diacylglycerol pyrophosphate; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; G-3-P, glycerol-3-phosphate; GFP, green fluorescent protein; LCAT, lecithin:cholesterol acyltransferase; LP, lipid particle(s); MBOAT, membrane-bound O-acyltransferase family; PAP, phosphatidate phosphatase; PDAT, phospholipid:DAG acyltransferase; PtdCho, phosphatidylcholine, PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; STE, steryl ester(s); TAG, triacylglycerol(s); VLCFA, very long chain fatty acids.

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Abstract

In the yeast *Saccharomyces cerevisiae* as in other eukaryotic cells non-polar lipids form a reservoir of energy and building blocks for membrane lipid synthesis. The yeast non-polar lipids, triacylglycerol (TAG) and steryl ester (STE), are synthesized by enzymes with overlapping function. Recently, genes encoding these enzymes were identified and gene products were partially characterized. Once formed, TAG and STE are stored in so-called lipid particles/droplets. This compartment which is reminiscent of mammalian lipoproteins from the structural viewpoint is, however, not only a lipid depot but also an organelle actively contributing to lipid metabolism. Non-polar lipid degrading enzymes, TAG lipases and STE hydrolases, also occur in redundancy in the yeast. These proteins, which are components of the lipid particle surface membrane with the exception of one plasma membrane localized STE hydrolase, mobilize non-polar lipids upon requirement. In this review, we describe the coordinate pathways of non-polar lipid synthesis, storage and mobilization in yeast with special emphasis on the role of the different enzymes and organelles involved in these processes. Moreover, we will discuss non-polar lipid homeostasis and its newly discovered links to various cell biological processes in the yeast.

Introduction

Lipid molecules are fundamental constituents of eukaryotic cells. The major classes of this group of biomolecules are phospholipids, sterols, sphingolipids and glycolipids. These lipids are the basis of biological membranes which protect the cell from the environment and constitute internal compartments. Other lipid species, however, form depots which can be mobilized upon requirement. Depending on the cell type, such storage lipids contain variable portions of mainly two lipid classes, namely sterols and fatty acids.

Sterols are major constituents of many biological membranes, where they serve as bulk components regulating fluidity and permeability, and also participate in the control of various membrane-associated processes (Yeagle et al., 1985; Parks and Cassey, 1995; Daum 1998; Umebayashi and Nakano, 2003; Sharma, 2006). Fatty acids are a source of cellular energy and also serve as building blocks for a number of complex membrane lipids. Both fatty acids and sterols have the capacity to act as signaling molecules and to affect gene expression via upstream promoter elements (Garbarino and Sturley, 2005). To circumvent the possible toxic and membrane disturbing accumulation of fatty acids and sterols and to conserve at the same time the chemical energy, excess amounts of these lipids are segregated within the cell in a modified and biologically inert form, namely as non-polar lipids triacylglycerols (TAG) and steryl esters (STE). These molecules lack charged groups and are therefore not suited as constituents of membrane bilayers. In contrast, these non-polar lipids are stored in specific compartments named lipid particles (LP), lipid droplets, lipid bodies or oil bodies. These subcellular organelles (Athenstaedt et al., 1999; Kent, 1995; Carman and Zeimetz, 1996; Zweytick et al., 2000; Brown, 2001) contain a hydrophobic core consisting of STE and TAG which is surrounded by a phospholipid monolayer. Moreover, LP contain a small set of specific proteins that are mainly involved in lipid metabolism.

When environmental conditions change, e.g., by depletion of nutrients forcing cells to make use of their stored resources, fatty acids from TAG and/or STE can either be used for energy production through β-oxidation or as building blocks for the synthesis of membrane lipids. The other degradation product formed by TAG hydrolysis, diacylglycerol (DAG), is also utilized as a substrate for phospholipid synthesis (Kent, 1995; Carman and Zeimetz, 1996; Carman and Henry, 1999; Voelker, 2000). Moreover, DAG can act as a second messenger in signal transduction upon binding to protein kinases (Becker and Hannun, 2005). The sterol moiety of STE can be directly incorporated into membranes or converted to the end product of the sterol biosynthetic pathway if present as a precursor sterol.

Biosynthetic routes leading to the formation of non-polar lipids and hydrolytic reactions catalyzing mobilization of these lipids are largely conserved from bacteria to humans. In this review article, we will focus on studies with the yeast *Saccharomyces cerevisiae* which is nowadays accepted as a reliable and most useful model system to study membrane and lipid biology. The reader is also referred to other recent reviews covering non-polar lipid biochemistry, molecular biology and cell biology (Turkish and Sturley, 2007; Daum, 2007; Czabany et al., 2007; Athenstaedt and Daum, 2006; Daum et al., 2007).

Biosynthesis of triacylglycerols

Eukaryotes make use of TAG, a fatty acyl ester derivative of glycerol, as the major storage variant for energy and fatty acids used as building blocks for membrane lipid biosynthesis. The high efficiency of TAG as an energy storage is based on its prevalent hydrophobic property enabling a tight packing in LP without affecting the polar hydrophilic environment (Turkish and Sturley, 2007; Wolins et al., 2006). An important precursor for the biosynthesis of TAG is phosphatidic acid (PtdOH). Depending on the utilization of either glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) as alternative

precursors, two principal biosynthetic pathways of PtdOH were identified in the yeast, namely the glycerol-3-phosphate (G-3-P) and the dihydroxyacetone phosphate (DHAP) pathway (Fig. 1). It has to be mentioned at this point that PtdOH is not only a precursor for TAG formation but also serves as a central metabolite in glycerophospholipid metabolism (Athenstaedt and Daum, 1999; Sorger and Daum, 2003). Moreover, PtdOH acts as a prominent regulator of lipid biosynthesis in the yeast. This regulation is achieved by the ability of PtdOH to bind and inactivate the transcriptional repressor Opi1p, thereby allowing derepression of transcription of various lipid biosynthetic enzymes when PtdOH accumulates (Loewen et al., 2004; Sreenivas et al., 2003; Wagner et al., 1999; Carman and Zeimetz, 1996; Greenberg and Lopes, 1996; Paltauf et al., 1992).

The predominant route of PtdOH synthesis in the yeast involves two acylation reactions starting with either glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) and activated fatty acids as precursors (Athenstaedt and Daum, 1999; Sorger and Daum, 2003). The first step of acylation leads to the formation of 1-acylglycerol-3-phosphate (lysophosphatidic acid; lysoPtdOH) and 1-acyl-DHAP, respectively. This initial step is carried out by stereospecific *sn*-1 acyltransferases. Zheng and Zou reported that Gat1p and Gat2p (Sct1p) from *Saccharomyces cerevisiae* are G-3-P/DHAP *sn*-1 acyltransferases with dual substrate specificity utilizing both G-3-P and DHAP as substrates. The contribution of Gat1p, which does not exhibit particular preference for acyl-CoA substrates, to the specificity of cellular fatty acid profiles, is only minor. Gat2p, on the other hand, with its apparent preference for C-16 saturated fatty acids plays a major role for the specific fatty acid composition of phospholipids (Zheng and Zou, 2001). Gat1p is the prevailing enzyme for the acylation of G-3-P/DHAP and dually localized to LP and the endoplasmic reticulum (ER). In contrast, localization of Gat2p is restricted to microsomes. Besides LP and the ER, G-3-P/DHAP acyltransferase activity was also found in mitochondrial fractions. Interestingly, this

yet unidentified acyltransferase(s) prefer(s) DHAP as a substrate for the acylation reaction (Zheng and Zou, 2001; Athenstaedt et al., 1999).

If 1-acyl-DHAP is formed as the first acylation product, it has to be reduced in an NADPH-dependent reaction prior to the second step of acylation. This reaction yielding lysoPtdOH is catalyzed by the acyl-DHAP reductase Ayr1p (Racenis et al., 1992; Athenstaedt and Daum, 2000). Similar to other enzymes of PtdOH biosynthesis, Ayr1p is dually located to LP and the ER. It appears, however, that in the latter compartment Ayr1p is not the only enzyme of this kind, although enzymes with overlapping function were not yet identified. It is worth mentioning that Ayr1p also exhibits a second enzymatic activity, namely that of 17β-hydroxysteroid dehydrogenase which is of interest with respect to fungal pathogenesis (Vico et al., 2002). Localization of a putative DHAP acyltransferase in mitochondria, and restriction of acyl-DHAP reductase(s) to LP and ER raises the question as to a possible interplay of organelles in this biosynthetic route. Access of the reductase(s) to acyl-DHAP may occur by partial solubility of the enzyme, or through membrane contact between the organelles which are involved (Athenstaedt and Daum, 2000).

In the final step of PtdOH biosynthesis from either G-3-P or DHAP, lysoPtdOH is converted to PtdOH by lysoPtdOH acyltransferases. The first yeast enzyme of this kind identified was Slc1p (sphingolipid compensation), which is homologous to a 1-acylglycerol-3-phosphate acyltransferase from *Escherichia coli* encoded by the *plsC* gene. Originally, the *SLC1* gene had been identified as a suppressor of a defect in sphingolipid biosynthesis. (Athenstaedt et al., 1999; Athenstaedt and Daum, 1997; Benghezal et al., 2007; Nagiec et al., 1993). Slc1p was localized to LP, but was also detected in the ER. According to biochemical and cell biological data, however, Slc1p was considered to be not the only yeast lysoPtdOH acyltransferase (Athenstaedt et al., 1999). Recently, indeed, a novel enzyme of this kind was discovered in the yeast by Benghezal et al. (Benghezal et al., 2007), Tamaki et al. (Tamaki et

al., 2007), Riekhof et al. (Riekhof et al., 2007) and Shilpa et al. (Shilpa et al., 2007) using different genetic and molecular biological approaches. These screenings led to the identification of the acyltransferase Slc4p (sphingolipid compensation), also known as Ale1p (acyltransferase for lyso-phosphatidylethanolamine) and Lpt1p (lysoPAF acyl-transferase), which is a member of the membrane-bound O-acyltransferase family (MBOAT). The four other MBOAT genes present in *S. cerevisiae* are *ARE1*, *ARE2*, *GUP1* and *GUP2*. The respective polypeptides harbor several transmembrane-spanning regions and share regions of sequence similarity (Benghezal et al., 2007; Tamaki et al., 2007; Riekhof et al., 2007; Shilpa et al., 2007). Slc4p/Ale1p/Lpt1p catalyzes transfer of acyl residues to various acceptors including lysoPtdOH, lysoPtdCho, lysoPtdIns, lysoPtdSer, lysoPtdGro and lysoPtdEtn (Tamaki et al., 2007). Most importantly, a synthetic lethal phenotype was shown for a *slc1*Δ *slc4* (*ale1*,*lpt1*)Δ double deletion emphasizing the indispensable role of these two gene products for PtdOH synthesis (Benghezal et al., 2007; Tamaki et al., 2007; Shilpa et al., 2007). However, the preference of Slc4p/Ale1p/Lpt1p to esterify lysophospholipids different from lysoPtdOH has been evidenced (Shilpa et al., 2007). Experiments using GFP-fusion proteins demonstrated that Slc4p/Ale1p/Lpt1p was localized to the ER (Tamaki et al., 2007; Huh et al., 2003) and found to be highly enriched in MAM (mitochondria associated membrane), an ER subfraction attached to the mitochondrial surface (Riekhof et al., 2007).

Ghosh et al. (personal communication; Rajasekharan R. Bangalore, India) showed that *YLR099C* (*ICT1*; Increased Copper Tolerance 1) encodes a novel, soluble acyl-CoA dependent lysoPtdOH acyltransferase which was highly expressed during organic solvent stress. Under these conditions, Ict1p contributes markedly to the biosynthesis of membrane phospholipids required for the repair of damaged membranes. A strain lacking *ICT1* showed reduced biosynthesis of major phospholipids both in the presence and in the absence of an organic solvent, and a dramatically reduced level of PtdOH.

The direct precursor of TAG synthesis is DAG (Daum et al., 2008) which is, however, also a precursor for aminoglycerophospholipid synthesis *via* the so-called Kennedy pathway and consequently a key intermediate in membrane lipid formation (Daum et al., 1998; Carman and Zeimetz, 1996; Carman and Henry, 1999; Voelker, 2000). DAG can be formed by dephosphorylation of de novo synthesized PtdOH; degradation of glycerophospholipids through the action of phospholipase C, or of phospholipase D in combination with phosphatidate phosphatase (PAP); and/or deacylation of TAG (Athenstaedt and Daum, 2006). Dephosphorylation of PtdOH catalyzed by PAP yields DAG and inorganic phosphate. Due to this function, PAP enzymes play an important role in lipid metabolism and formation/degradation of lipid signaling molecules in eukaryotic cells. In the yeast, the major PAP activity has recently been attributed to the gene product of *PAH1* (phosphatidic acid phosphohydrolase). This polypeptide contains a DxDx(T/V) catalytic motif which is shared with the mammalian fat-regulating protein lipin 1, the mammalian counterpart of Pah1p (Han et al., 2006; Donkor et al., 2007) and the superfamily of haloacid dehalogenase-like proteins (Han et al., 2006). Pah1p belongs to the PAP1 group of enzymes, which are characterized by their requirement of Mg^{2+} as a cofactor for catalytic activity (Han et al., 2006; Carman and Han; 2006) and appears to be a key regulator in transcription of phospholipid biosynthetic genes and nuclear/ER membrane growth (Santos-Rosa et al., 2005). Pah1p is associated with both the membrane and cytosolic fractions of the cell. The enzyme activity is regulated by lipids (Wu et al., 1996; Wu et al., 1993) nucleotides (Wu and Carman, 1994) and phosphorylation (Santos-Rosa et al., 2005; O´Hara et al., 2006). This complex network of regulatory mechanisms appears to enable cells coordinating synthesis of phospholipids via the CDP-DAG pathway and synthesis of TAG (Carman, 1997; Carman and Zeimetz, 1996).

Since *pah1Δ* mutants still contain some PAP activity, the presence of additional genes encoding PAPs in the yeast was anticipated. It was shown that the yeast gene products of

DPP1 and *LPP1* also contributed to this enzymatic reaction. Dpp1p and Lpp1p are members of PAP2 class enzymes which are characterized by their Mg^{2+} independence. Almost the entire PAP2 activity in the yeast is derived from Dpp1p and Lpp1p with the former enzyme being the predominant contributor (Carman and Han, 2006). In addition to PtdOH, both enzymes use DAG pyrophosphate (DGPP), lysoPtdOH, sphingoid base phosphates and isoprenoid phosphates as substrates (Toke et al., 1998; Toke et al., 1998; Wu et al., 1996; Furneisen and Carman, 2000; Faulkner et al., 1999). Dpp1p and Lpp1p are integral membrane proteins with six transmembrane spanning regions and localized to the vacuole and the Golgi compartment. The two proteins share homologous regions which constitute a three-domain lipid phosphatase motif localized to the hydrophilic surface of the membrane. This catalytic motif is shared with a superfamily of Mg^{2+} independent lipid phosphatases (Toke et al., 1998; Toke et al., 1998; Stukey and Carman 1997; Han et al., 2001). The lipid phosphatase activities of Dpp1p and Lpp1p were assumed to play a role in phospholipid metabolism and cell signaling by controlling the relative amounts of DGPP, other lipid phosphates and their dephosphorylation products (Furneisen and Carman, 2000).

It should also be mentioned that yeast can generate PtdOH through the breakdown of phosphatidylcholine (PtdCho) or phosphatidylethanolamine (PtdEtn) catalyzed by hydrolases of the phospholipase D type, e.g. Pld1p (Rose et al., 1995; Waksman et al., 1996; Waksman et al., 1997; Ella et al., 1996; McDermottet al., 2004). Alternatively, phosphorylation of DAG by DAG kinase may also lead to the formation of PtdOH. This reaction, however, and the respective enzyme(s) have never been identified in the yeast. Finally, DAG can also be formed from TAG by TAG lipases (see below) or from phospholipids through the action of a phospholipase C, e.g., Plc1p (Flick and Thorner, 1993).

The final step of TAG synthesis (see Fig. 1), acylation of DAG in the *sn*-3 position, can be accomplished by two different types of reactions, one being acyl-CoA dependent and

the other acyl-CoA independent. In the yeast, DAG esterification is mediated by four enzymes with different structural properties. The major enzyme catalyzing acyl-CoA dependent DAG acylation is the acyl-CoA:diacylglycerol acyltransferase (DGAT) Dga1p (Lehner and Kuksis 1996; Sorger and Daum, 2002). This enzyme belongs to the family of *DGAT2* (diacylglycerol acyltransferase) proteins, which harbor transmembrane regions and lack classical signal peptides. This family of proteins typically also features motifs and residues implicated at the active sites of bacterial glycerol-3-phosphate acyltransferases (Lewin et al., 1999; Oelkers et al., 2002). Dga1p preferentially utilizes oleoyl-CoA and palmitoyl-CoA as substrates, but myristoyl-CoA, stearoyl-CoA, arachidonyl-CoA and linoleoyl-CoA are also accepted in vitro although with minor efficiency (Oelkers al., 2002). Enzyme assays using different subcellular fractions from a *dga1Δ* yeast deletion mutant revealed localization of Dga1p to LP and the ER. In the former compartment, Dga1p appeared to be the only acyl-CoA-dependent DGAT, whereas additional enzymes catalyzing TAG synthesis seemed to be present in microsomal fractions (Sorger and Daum, 2003). As will be described below, these activities were attributed to the STE synthases Are1p and Are2p.

The second major reaction leading to TAG synthesis in the yeast is catalyzed by Lro1p (LCAT-related open reading frame), a homologue of the human LCAT (lecithin:cholesterol acyltransferase). Lro1p converts DAG to TAG in an acyl-CoA independent esterification reaction using the *sn*-2 acyl group from glycerophospholipids, preferentially PtdCho and PtdEtn, as co-substrate for the third acylation step. Consequently, Lro1p was identified as a phospholipid:DAG acyltransferase (PDAT) (Dahlqvist et al., 2000; Oelkers et al., 2000). The activity of this enzyme appeared to be restricted to the ER. Dioleoyl-PtdEtn and dioleoyl-PtdCho were preferentially used as co-substrates. Ergosterol was not accepted as a substrate as one might have expected for a homologue to the human LCAT (Dahlqvist et al., 2000). Whether acylation of DAG is catalyzed by Dga1p and/or Lro1p largely depends on the

growth phase of the yeast. Whereas Lro1p is believed to be mainly active in the logarithmic growth phase, Dga1p activity is more pronounced in the stationary phase (Oelkers et al., 2000).

In addition to the major pathways of TAG biosynthesis described above, alternative routes of TAG synthesis exist in the yeast. The two STE synthases, Are1p and Are2p, also catalyze TAG synthesis although with minor efficiency (Sorger et al., 2004). They are responsible for residual DGAT activity in the ER when both *LRO1* and *DGA1* are deleted. Are1p and Are2p are members of the DGAT1 family and thus different from the acyl-CoA dependent acyltransferase Dga1p which belongs to the DGAT2 family (Lardizabal et al., 2001; Cases et al., 2001). In contrast to Dga1p, Are1p and Are2p readily utilize linoleoyl-CoA as substrate whereas palmitoyl-CoA is barely incorporated into TAG (Yang et al., 199 7). *DGA1* unlike the *ARE* genes is transcriptionally up-regulated during growth to saturation and nitrogen deprivation (Oelkers et al., 2002). The finding that all acyltransferases contributing to TAG formation are localized to the ER (with additional presence of Dga1p in LP) clearly identified this organelle as the major site of TAG synthesis. How non-polar lipids synthesized in the ER are incorporated into their site of storage, the LP, still needs to be elucidated.

Using molecular biological methods, non-oleaginous *Saccharomyces cerevisiae* has been changed to oleaginous yeast with approximately 30% lipid content thus allowing investigations on mechanisms of lipid accumulation in some detail. Kamisaka et al. (Kamisaka et al., 2007) found that overexpression of *DGA1* and enhanced leucine biosynthesis caused lipid accumulation in a *snf2*Δ deletion strain much more pronounced than in wild type. *SNF1* encodes a transcription factor forming part of the SWI/SNF chromatin remodeling complex. In contrast to *DGA1*, *LRO1* overexpression in the *snf2*Δ mutant background decreased total amounts of fatty acids and cellular lipids, whereas a slight

increase in total fatty acids and lipids was observed upon *LRO1* overexpression in wild type. Since *snf2*Δ is known to markedly decrease the expression level of genes encoding phospholipid biosynthetic enzymes that are regulated by inositol and choline (Kodaki et al., 1995), the amount of phospholipid co-substrates for Lro1p may not be sufficient in the *snf2*Δ strain. Overexpression of *DGA1* in the *snf2*Δ disruptant significantly increased DGAT activity which may contribute to the observed lipid accumulation. However, the enhanced DGAT activity in strains overexpressing *DGA1* in the *snf2*Δ mutant background was not due to increased amounts of Dga1p (Kamisaka et al., 2007; Kamisaka et al., 2006). This observation led to the assumption that *DGA1* was activated by post-translational modifications as described previously (Coleman and Lee, 2004; Lung and Weselake, 2006).

Biosynthesis of steryl esters

STE formation involves reaction of a fatty acid molecule with the hydroxyl group of sterols (Garbarino and Sturley, 2005). In eukaryotic cells, esterification of sterols is highly important to maintain sterol homeostasis. STE formation can be accomplished by two different enzymatic mechanisms. First, an acyl-CoA-independent reaction is catalyzed by enzymes of the lecithin:cholesterol acyltransferase (LCAT) type which use phospholipids as acyl donors. Such a reaction, however, has never been demonstrated for the yeast. Secondly, acyl-CoA:cholesterol acyltransferases (ACAT) form STE from sterols and activated fatty acids in an acyl-CoA-dependent reaction. In *Saccharomyces cerevisiae*, two acyl-CoA:sterol acyltransferases catalyzing the synthesis of STE were identified, namely Are1p and Are2p (ACAT related enzymes) (Fig. 2). Sterol esterification is completely abolished when both STE synthases are deleted suggesting that these two gene products were the only yeast STE biosynthetic enzymes. Surprisingly, the *are1Δ are2Δ* double mutant did not exhibit an obvious growth phenotype (Oelkers and Sturley, 2004; Yu et al., 1996). As mentioned already

in the previous section, both STE synthases, particularly Are2p, have a limited capacity to esterify DAG (Müllner and Daum, 2004).

Are1p and Are2p are 49% identical to each other and exhibit approximately 24% identity to human ACAT (Yu et al., 1996; Yang et al., 1996). The half-life of the *ARE2* mRNA is 12 times longer than that of the *ARE1* transcript (Jensen-Pergakes et al., 2001). The major ACAT enzyme activity was attributed to Are2p accounting for at least 65-75% of total STE synthase activity as estimated by *in vitro* assays (Yu et al., 1996). Both STE synthases are localized to the same subcellular compartment, the ER, but are different with respect to their product formation. Are1p, although esterifying ergosterol at reasonable amount, exhibits some preference for precursor sterols as substrates, particularly lanosterol (Zweytick et al., 2000). This finding is in agreement with the view that Are1p may prevent excessive accumulation of sterol intermediates. Consequently, Are1p forms depots of these sterol intermediates in the form of STE that can be rapidly utilized to enter the sterol biosynthetic pathway upon urgent requirement. It was assumed that ergosterol may be formed more efficiently and faster from sterol intermediates liberated from STE than through *de novo* sterol synthesis. Such a route of ergosterol formation also circumvents the early steps of the sterol biosynthetic pathway which may be blocked or down regulated under certain conditions. Recent experiments from our laboratory (A. Wagner et al., manuscript in preparation) demonstrated that under ergosterol depletion caused by poisoning cells with terbinafine precursor sterols from STE were indeed rapidly mobilized and converted to the end product of the ergosterol biosynthetic route. The vast majority of cellular ergosteryl esters is formed by Are2p, providing an important sterol pool for membrane formation (Zweytick et al., 2000).

Overexpression of *ARE1* or *ARE2* did not exhibit a marked effect on the composition of the free sterol fraction, as ergosterol remained the major sterol produced. Taking a closer insight into the emerging effect on STE formation two partially divergent opinions are to be

mentioned. Polakowski et al. (Polakowski et al., 1999) did not ascertain a noticeable increase in esterified sterols when overexpressing *ARE1*, neither by increasing the gene copy number nor by using a strong promoter. Substrate limitation was proposed to be responsible for this lack of enhanced steryl ester formation (Yang et al., 1996). By contrast, an alternative view is being advanced by Jensen-Pergakes (Jensen-Pergakes et al., 2001), claiming that the ester fraction of cells overexpressing *ARE1* shows a marked accumulation of sterol intermediates, specifically lanosterol and, to a lesser extent, zymosterol. Cells overexpressing *ARE2* preferentially esterified ergosterol (Jensen-Pergakes et al., 2001). Interestingly, all sterols that are positively affected by *ARE2* overexpression are able to replace ergosterol as a membrane constituent (Nes et al., 1993) and may therefore represent a set of “first aid” components. The substrate specificity of Are2p with its preference for ergosterol and specific ergosterol precursors correlates with the decreased specificity *in vitro* for lanosterol compared to ergosterol (Jensen-Pergakes et al., 2001).

The contribution of the two yeast STE synthases to sterol esterification is subject to individual regulation. An example for the differential regulation of *ARE1* and *ARE2* is their dependence on the heme status and the oxygen supply of the yeast. Are1p and Are2p represent an enzyme pair that appears to have an adjusting capacity to the oxygen status of the yeast cell. Are1p is the hypoxic form among the two STE synthases, which is induced by heme depletion, whereas Are2p represents the major aerobic activity in heme-competent cells. The presence of heme-regulated elements in gene promoters and the effect of heme on STE levels suggested that transcriptional regulation by heme might be involved in the response of sterol esterification to the presence or absence of oxygen. The *ARE1* transcript was abundant in heme-depleted cells and significantly reduced after induction of heme synthesis. On the contrary, the low level of the *ARE2* transcript in heme-depleted cells was markedly increased when heme synthesis was induced. The transcriptional level of both genes was extremely low

in cells grown in the absence of oxygen (Valachovic et al., 2002). These results indicate that transcriptional regulation of *ARE* genes by heme combined with the substrate specificities of Are1p and Are2p may be involved in the adaptation of yeast sterol metabolism to hypoxia.

In *are1Δ are2Δ* double deletion strains the amount of total sterols is reduced to about 80% of free and esterified sterols in wild type cells. It appears that STE synthesis is tightly linked to sterol synthesis and involved in its regulation. First, in strains deleted of both *ARE1* and *ARE2* the flux of precursor sterols is slightly affected which results in changes of the total sterol pattern in such mutant strains (Czabany et al., manuscript in preparation). This type of regulation appears to be related rather to changes in the flux of intermediates in the sterol biosynthetic pathway than to transcriptional or translational regulation of enzyme formation. Secondly, STE synthesis deficient strains showed specific down-regulation of *ERG3* encoding an enzyme of the late ergosterol biosynthetic pathway (Arthington-Skaggs et al., 1996). *ERG3* was shown to be subject to regulation by mutations in the ergosterol biosynthetic pathway which alter intracellular sterol composition, and by mutations in the *ARE* genes which alter the ratio of free to esterified sterols (Arthington-Skaggs et al., 1996). It was also demonstrated that two transcription factors, Upc2p and Ecm22p, bind to promoters of most ergosterol biosynthetic genes including *ERG2* and *ERG3*, and activate these genes upon sterol depletion. Despite strong C-terminal sequence similarities, Upc2p and Ecm22p respond differently to an increased requirement for sterols (Davies et al., 2005).

Regulation of sterol homeostasis appears to occur not only at the enzymatic level, but also at the organelle level. As demonstrated by Leber et al. (Leber et al., 1998) the yeast squalene epoxidase Erg1p is dually located to the ER and LP, but enzymatic activity could only be shown *in vitro* with isolated microsomal fractions. It was argued that a reductase present only in the ER may be the missing cofactor in LP. Erg1p is known to exhibit only low specific activity and is therefore a rate-limiting enzyme in ergosterol biosynthesis (M´Baya et

al., 1989). Thus, embedding of enzymatically inactive Erg1p in LP may provide an additional regulatory possibility of total cellular enzyme activity at the organelle level. In higher eukaryotes, it was demonstrated that a cytosolic protein participated in the regulation of squalene epoxidase (Shibata et al., 2001). Although there is no proof for such a regulatory mechanism in the yeast, involvement of LP in the regulation of squalene epoxidase activity may be considered (Sorger et al., 2004).

When both *ARE1* and *ARE2* are deleted synthetic lethality is brought about by an additional mutation in the *ARV1* (*ARE2* required for viability) gene (Tinkelenberg et al., 2000). *ARV1* was predicted to encode a transmembrane protein that possesses potential zinc-binding motifs. It was proposed that Arv1p is involved in sterol trafficking, especially in mediating sterol exit from the ER which becomes essential in the absence of the neutralizing effect of sterol esterification (Kamisaka et al., 2006; Tinkelenberg et al., 2000). Arv1p, however, appears to play not only a pivotal role in sterol homeostasis but also affects yeast sphingolipid metabolism (Swain et al., 2002). Cells lacking Arv1p displayed an altered intracellular sterol distribution and were defective in sterol uptake, consistent with a role of Arv1p in sterol trafficking. Human *ARV1*, a predicted sequence ortholog of the yeast *ARV1*, complemented the defects in an *arv1Δ* yeast mutant (Tinkelenberg et al., 2000).

Non-polar lipid storage in lipid particles

All eukaryotes are able to store energy in the form of TAG either alone or in combination with STE. Normally, the excess of TAG and STE is stored in distinct organelles called lipid particles (LP), also known as lipid droplets, oil droplets, lipid bodies, oil bodies or oleosomes in mammals and plants, respectively (Jayaram and Bal, 1991; Wach, 1996). The hydrophobic core of these compartments is surrounded by a phospholipid monolayer membrane equipped with a small amount of proteins (Zweytick et al., 2000). In mammalian

cells, lipid droplets are rich in TAG and/or cholesteryl esters with a small amount of proteins embedded in their phospholipid surface monolayer. Lipid droplets of higher eukaryotes are unique with their most abundant proteins called perilipin in mammals or oleosin in plants (Greenberg et al., 1991; Fujimoto et al., 2004; Qu et al 1990). Perilipin plays an important role in regulation of basal and hormonally stimulated lipolysis in vertebrates. During energy depletion, a cAMP-dependent protein kinase phosphorylates perilipin and facilitates lipolysis by hormone-sensitive lipase and adipose TAG lipase. Under basal conditions, perilipin binds to a protein called CGI-58 and enhances TAG storage by limiting hydrolysis of TAG (Yamaguchi et al., 2007; Subramanian et al., 2004; Yamaguchi et al., 2004; Lefevre et al., 2001). In addition to perilipin, mammalian lipid droplets contain further structural proteins such as adipophilin, TIP47 (Tail-Interacting Protein 47 kDa), S3-12 and OXPAT, also known as Lipid storage Droplet Protein (Brown and Goldstein, 1976; Brown et al., 1975).

Clausen et al. (Clausen et al., 1974) showed that the hydrophobic core of LP from *Saccharomyces cerevisiae* consists of TAG and STE in an approximately equal ratio with more unsaturated fatty acids in STE. The standard diameter of LP in wild type yeast cells ranges from 0.3 to 0.5 µm (Leber et al., 1994). In strains producing only one type of non-polar lipid, TAG or STE, respectively, the situation slightly changes. Recent work in our laboratory (T. Czabany and A. Wagner, unpublished data) showed that in an *are1*Δ *are2*Δ *lro1*Δ triple deletion strain the diameter of LP containing only TAG derived from Dga1p was 250 to 750 nm. A largely unaltered size distribution of LP was observed in strains with only Lro1p or one STE synthase, Are1p or Are2p, active. These results suggested that any single acyltransferase is sufficient for the formation of yeast LP, and Dga1p may play a crucial role in the size distribution of LP. In the same study, the internal structure and organization of lipids in LP from *Saccharomyces cerevisiae* was analyzed using differential scanning calorimetry and X-ray small angel scattering. It was observed that STE form outer layers and TAG the inner part

of the hydrophobic core of LP. STE containing LP showed ordered structures whereas LP from strains containing only TAG did not. In contrast to *Saccharomyces cerevisiae*, LP from the yeast *Yarrowia lipolytica* vary strongly in size, properties and composition (fatty acids and proteins) as a results of the carbon source used for cultivation (Blanchette-Mackie et al., 1995).

Biogenesis of lipid particles

Lipid particles are believed to originate from membranes of the ER. Several models of LP biogenesis were postulated in the literature. These models include budding of ER domains enriched in non-polar lipids ("nascent LP"); incorporation of lipid/protein complexes into preexisting LP; and delivery of proteins and lipids to preexisting LP through vesicle transport. The hypothesis which is currently favored by many laboratories is LP formation through budding from the ER. This model suggests that non-polar lipid synthesizing enzymes segregate in a lens-like pool between the luminal and cytosolic leaflets of the ER which leads to an accumulation of non-polar lipids in this specific region (Blanchette-Mackie et al., 1995; Robenek et al., 2006; Scow et al., 1980). Since non-polar lipids cannot be integrated into the phospholipid bilayer of the ER at substantial amounts, they are believed to form a hydrophobic micro-droplet between the two leaflets of the ER membrane. As hydrophobic domains in the ER membrane grow, they get engulfed by a monolayer membrane with certain proteins embedded and finally bud off the ER. There is experimental evidence behindhand to support this model. First, biochemical and cell biological experiments demonstrated that major acyltransferases forming TAG and STE are indeed localized to the ER. Secondly, LP are surrounded by a monolayer membrane, and proteins lacking transmembrane domains are favorably embedded. Thirdly, LP and the ER share a certain set of proteins. Finally, newly

formed LP can be found in proximity to the ER (Blanchette-Mackie et al., 1995; Cohen et al., 2004).

Although substantial evidence supports the view that the non-polar lipid synthesizing machinery defines the origin of LP from the ER, there is no direct evidence explaining targeting of proteins from the ER to LP. Neither specific signal sequences nor targeting domains have so far been identified in yeast LP proteins. The only molecular property which LP seem to have in common is the presence of one or two hydrophobic domains near the C-terminus (Müllner et al., 2004). When these hydrophobic domains were removed from the typical LP proteins Erg1p, Erg6p and Erg7p, subcellular targeting of these proteins was affected insofar as substantial portions of the truncated polypeptides were found in the ER membrane. These results suggested that ER and LP are related organelles.

In wild type yeast cells, Erg7p is one of the prominent LP proteins and localized more or less exclusively to this compartment. In contrast, Erg1p is approximately equally distributed between ER and LP, and Erg6p and Ayr1p are also dually localized to the two subcellular fractions although with distinct preference for LP (Sorger et al., 2004). To address the question as to the fate of typical LP proteins in a strain which does not contain LP, experiments were performed using a *dga1*Δ *lro1*Δ *are1*Δ *are2*Δ quadruple mutant. Deletion of these genes led to a defect in LP biogenesis in yeast (Sorger et al., 2004) and resulted in retention of Erg1p, Erg6p, Erg7p and Ayr1p to ER membranes. When the quadruple mutant was transformed with plasmids bearing inducible *DGA1*, *LRO1*, or *ARE2*, an efficient LP proliferation and redirection of Ayr1p and Erg6p from microsomes to the newly formed LP occurred. These results confirmed the close relationship between LP and ER, and also supported the assumption that these proteins have left the bilayer environment of the ER along with an emerging core of non-polar lipids during the process of LP biogenesis.

According to another model of LP biogenesis (Huang, 1992), proteins of this organelle can be targeted to their destination with the help of targeting signals through vesicle flux. The yeast gene product of *YBR177c* contains a region which is homologous to the putative human membrane receptor HPS1. This receptor had been shown to facilitate the process of vesicle docking for the biogenesis of LP in mammalian cells (Athenstaedt et al., 1999). The homology between the two polypeptides led to the assumption that the YBR177c protein may also have the capacity to interact with targeting signal sequences, and thus was considered contributing to protein targeting through vesicle flux. However, a yeast mutant deleted of *YBR177c* contained normal LP and did not show any defect in LP biogenesis. Therefore, this model of yeast LP formation remains hypothetical at present.

The several hypotheses describing LP biogenesis led to the speculation that there is more than one mechanism contributing to this process. Consequently, one would not expect that all LP proteins have properties in common. This view is supported by the recent findings (Ploegh, 2007; Welte, 2007) that LP act as intermediate organelles accumulating ubiquitinated proteins to be destroyed by the cellular degradation machinery, but may also serve as vehicles to transport proteins to other organelles like mitochondria, ER and peroxisomes. Since LP appear to be in close contact to these organelles, they could recruit or supply proteins and macromolecules through different sequestration mechanisms. Such cellular strategies may explain why formation of LP may be affected by variable physiological conditions of the cell.

Catabolism of non-polar lipids

Originally, LP were considered only as a storage organelle for non-polar lipids. This view has changed during the last decade when various proteome analyses of LP from different cellular sources were performed. Recent discoveries demonstrated that yeast LP and those from other cell types are also actively involved in lipid metabolism including reactions of

TAG synthesis (Benghezal et al., 2007; Sorger and Daum, 2002; Sorger et al., 2004), PA synthesis (Benghezal et al., 2007), sterol biosynthesis (Athenstaedt et al., 1999; Sorger et al., 2004; Leber et al., 1998; Müllner et al., 2004; Milla et al., 2002), fatty acid activation (Johnson et al., 1994), but also in TAG and STE mobilization (Athenstaedt and Daum, 2003; Kurat et al., 2005). Mobilization of the energy rich hydrophobic core of LP plays a key role at various stages of yeast cell growth, especially during starvation, meiosis and sporulation. Hydrolysis of lipid depots is very rapid especially when cells from the stationary phase are shifted to fresh culture medium (Gray et al., 2004). Upon requirement or nutrient depletion lipases and hydrolases mobilize stored TAG and STE, respectively, to ensure the availability of fatty acids, sterols and DAG from lipid depots. In recent years, three yeast TAG lipases were identified and shown to be localized to LP (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005) (Fig. 3). Similarly, three yeast STE hydrolases were identified and partially characterized. Two STE hydrolases were localized to LP, but the major STE hydrolase activity was found in the plasma membrane (Zinser et al., 1993). Notably, STE degradation plays a key role in cellular sterol homeostasis.

Triacylglycerol mobilization

TAG is the most efficient energy source in biological systems and considered to be of minor toxicity in the physiological sense. Degradation of TAG in the yeast *Saccharomyces cerevisiae* is catalyzed by three different lipases which are encoded by *TGL3*, *TGL4* and *TGL5* (Athenstaedt and Daum, 2003; Athenstaedt and Daum, 2005). The corresponding gene products are embedded in the phospholipid surface monolayer of LP. Among these three lipases, Tgl3p was found to exhibit the highest TAG hydrolytic activity *in vitro* and *in vivo* although with less specificity towards acyl chain length than the other TAG lipases.

Tgl3p was the first yeast TAG lipase identified at the molecular level and by function (Athenstaedt and Daum, 2003). A mutant strain lacking the gene product of *YMR313c* (Tgl3p) showed an elevated level of cellular TAG. Mass spectrometric and computational analysis demonstrated that Tgl3p contains the consensus sequence motif GXSXG which is typical for lipolytic enzymes. Secondary structure analysis of Tgl3p revealed the presence of hydrophobic stretches at the C-terminus of the protein and the lack of transmembrane spanning domains. The subcellular localization of Tgl3p to LP was shown by using hybrids N-terminally tagged with green fluorescent protein (GFP) (Athenstaedt and Daum, 2003; Kurat et al., 2005) and confirmed by enzymatic analysis using isolated subcellular fractions from the yeast.

Mobilization of TAG can be measured *in vivo* when cells are poisoned with cerulenin, an inhibitor of yeast fatty acid synthesis. In the presence of cerulenin, a *tgl3*Δ deletion strain mobilized TAG less efficiently than wild type and became hypersensitive to the drug. Overexpression of *TGL4* under control of a *GAL1/10* promoter in *tgl3*Δ *tgl4*Δ double mutants showed an increased level of DAG up to 12% of total cellular lipids, but *TGL3* overexpression in the same mutant background rescued this defect reducing the level of DAG to 6% of total cellular lipids (Kurat et al., 2005). The fatty acid analysis of TAG stored in the *tgl3*Δ deletion strain showed an elevated level of C14:0, C16:0 and C26:0 fatty acids (Athenstaedt and Daum, 2003). Altogether, these results suggested that Tgl3p is responsible for the major lipolytic activity in LP and whole cells and preferentially utilizes TAG and DAG containing medium chain and very long chain fatty acids as substrates *in vivo.*

Since TAG lipase activity was not completely abolished in a *tgl3*Δ deletion strain, the presence of additional TAG lipase(s) was anticipated (Athenstaedt and Daum, 2003). Homology searches and multiple sequence alignments led to the identification of genes encoding the additional yeast TAG lipases Tgl4p and Tgl5p. Even though all three lipases do

not show great homology towards other lipases identified so far, they have the common conserved sequence motive GXSXG. Their catalytic domains consist of a mainly parallel eight β-sheet structure connected by helical loops of various lengths (Brady et al., 1999; Ollis et al., 1999; Schrag et al., 1997). Microscopic analysis of strains bearing Tgl4p-GFP and Tgl5p-GFP hybrids verified the subcellular localization of Tgl4p and Tgl5p in LP (Kurat et al., 2005; Athenstaedt and Daum, 2005). The double deletion strain *tgl4*Δ *tgl5*Δ accumulated 1.7 fold more TAG than wild type. However, a single deletion of *tgl5*Δ did not show reduced TAG hydrolysis *in vivo* compared to wild type in the presence of cerulenin, but an elevated level of very long chain fatty acids (VLCFA) in TAG. The amount of TAG stored in the *tgl4*Δ *tgl5*Δ double deletion strain was markedly higher than in the *tgl4*Δ single mutant. These results suggested an additive effect of Tgl4p and Tgl5p with Tgl5p probably serving as a regulator or modulator of TAG hydrolysis.

Most recently, it was demonstrated that serine 315 in the GXSXG lipase active site of the patatin domain from Tgl4p is essential for its hydrolytic activity, and Tgl4p is an ortholog of the mammalian ATGL (Kurat et al., 2005). In addition, Tgl4p was recognized as a physiological substrate of Cdc28/CDK1 (Ubersax et al., 2003). The same patatin-like acylhydrolase domain was also identified in a lipase from *Arabidopsis thaliana* (Eastmond, 2006). These findings suggested that the mechanism of lipolysis is evolutionarily conserved among eukaryotes (Kurat et al., 2005). It has to be noted, however, that in contrast to the yeast TAG lipases some animal and plant TAG lipases showed also phospholipase activity (Durand et al., 1978; Bhardwaj et al., 2001).

Even though Tgl3p, Tgl4p and Tgl5p are localized to the same subcellular compartment, the LP, and known to hydrolyze TAG and DAG exclusively, they have some degree of specificity towards TAG molecules with certain fatty acids (Athenstaedt and Daum 2005). Tgl4p showed some preference towards C14:0 and C16:0 containing TAG, whereas

the level of the VLCFA C26:0 stored in TAG from *tgl3Δ* and *tgl5Δ* was markedly elevated. Generally, VLCFA are indispensable for sphingolipid biosynthesis and crucial for raft association in yeast (Smith and Lester, 1974; Oh et al., 1997). To understand the link between TAG lipolysis and sphingolipid metabolism, we recently carried out experiments labeling major sphingolipids and phospholipids from wild type and *tgl* mutants with [^{3}H]inositol and [^{14}C]palmitic acid as precursors (S. Rajakumari, unpublished results). We observed that *tgl* mutants had a markedly reduced rate of sphingolipid biosynthesis and an increased rate of sphingolipid turnover compared to wild type. In addition, *tgl* deletion mutants harbored defects in phospholipid biosynthesis. These findings strongly suggested that lipolysis rate and substrate specificity of the three TAG lipases affect yeast sphingolipid and phospholipid metabolism. These experiments, however, are only a starting point to understand the physiological role of yeast TAG lipases. Despite the progress that has been made to elucidate the function of these enzymes, their precise membrane topography, regulation and substrate accessibility await further investigation.

In addition to the three yeast TAG lipases identified by function so far, a gene product encoded by *TGL2* has been described in the literature (Van Heusden et al., 1998). This protein, which was considered a potential TAG lipase by sequence homology, turned out to exhibit no such enzyme activity in the yeast. In contrast, expression of the *TGL2* gene in *E. coli* bearing a defect in DAG kinase could increase the hydrolysis of TAG and DAG with short-chain fatty acids and complement the defect in and *E. coli* DAG kinase disruptant. The role of Tgl2p in yeast lipid metabolism is still ambiguous.

Steryl ester hydrolysis

STE serve as major storage form of fatty acids and sterols. Both components are important constituents of biological membranes. Regulation of STE metabolism is especially

important to maintain sterol homeostasis within a cell. A flexible STE pool tentatively contributes to defend the cell against both a surplus and a lack of free sterols (Brown et al., 1980). Consequently, STE hydrolytic enzymes play an important role in the maintenance of cellular sterol levels. In *Saccharomyces cerevisiae*, three STE hydrolases were identified, namely Yeh1p, Yeh2p and Tgl1p. Tgl1p and Yeh1p were localized to LP, whereas Yeh2p was detected in the plasma membrane (Zinser et al., 1993; Taketani et al., 1978).

Taketani et al. (Taketani et al., 1978) had shown many years ago that the yeast mitochondrial fraction prepared from aerobic cells catalyzed STE hydrolysis. However, purity of fractions used in this study was not comparable to standards of more recent work. In contrast, Zinser et al. (Zinser et al., 1993) showed that the maximum yeast STE hydrolase activity was present in the plasma membrane. Screening of the genome of *Saccharomyces cerevisiae* for putative STE hydrolases on the basis of known sequences of mammalian STE hydrolases led to the identification of *YEH2* (Müllner et al., 2005; Köffel et al., 2005). Deletion of this gene caused a defect in STE hydrolase activity only in the plasma membrane accompanied by a slight increase in zymosterol (Müllner et al., 2005). Localization of Yeh2p in the plasma membrane was confirmed by using GFP hybrid proteins. An N-terminally His-tagged hybrid of Yeh2p showed high activity *in vivo* and *in vitro* and preferentially utilized zymosteryl esters as a substrate. The enhanced enzymatic activity of the His-Yeh2p fusion protein may be due to the “affirmative” change in the active site of the enzyme. Overexpression of this hybrid protein altered the plasma membrane structure and sensitivity of cells towards various drugs and stress.

In addition to Yeh2p, two other yeast STE hydrolases were identified as paralogues of mammalian acid lipases (Köffel et al., 2005), namely Tgl1p and Yeh1p. Fluorescence microcopy showed that a C-terminally tagged Tgl1p-GFP hybrid localizes to LP. Deletion of *TGL1* affected hydrolysis of STE *in vivo* and led to a two fold increase of the STE to

phospholipid ratio, whereas the TAG level remained unaltered. Not surprisingly, deletion of *TGL1* did not eradicate STE hydrolysis activity. Examination of substrate specificity using fluorescently labeled lipid analogues confirmed that Tgl1p preferentially hydrolyzed STE compared to TAG. In another study, however, it was shown that Tgl1p exhibited STE hydrolase activity at pH 4.5-7.4, and low TAG lipase activity at pH 7.4 *in vitro* (Jandrositz et al., 2005).

Mobilization of STE was also examined in a *yeh1*Δ *yeh2*Δ *tgl1*Δ triple mutant and *yeh1*Δ *tgl1*Δ and *yeh2*Δ *tgl1*Δ double mutants in a heme-deficient strain background (Köffel and Schneiter, 2006). Under these conditions, the triple mutant and the *yeh1*Δ *tgl1*Δ double deletion strain showed a marked defect in STE catabolism. It became evident from these studies that the function of Yeh1p is essential to mobilize STE efficiently during anaerobic conditions which are mimicked by heme deficiency in aerobically cultivated cells. Moreover, the steady state level of *YEH1* expression was increased 1.5 fold when cells were cultivated in the presence of cholesterol. This up-regulation of *YEH1* required Rox3p, a component of the mediator complex which affects transcription through RNA polymerase II in *Saccharomyces cerevisiae*. Deletion of *ROX3* caused a reduced level of Yeh1p under anaerobic conditions. Thus, the differential regulation of the three STE hydrolases may be explained by the different response to the mediator component Rox3p at the transcriptional level.

Summary, conclusions and future directions

In recent years, investigations of non-polar lipid metabolism gained increasing interest due to the link of lipid storage to health and disease. The evidence, that has been accumulated, addressed biochemical, physiological and more recently molecular biological and cell biological aspects of this topic. An experimental model system which contributed especially

to the molecular understanding of non-polar lipid metabolism and storage is the yeast *Saccharomyces cerevisiae* due to its well known advantages such as ease of manipulation by nutritional and genetic means. Yeast research led to a number of important results in the field of TAG and STE synthesis, degradation and depot formation which were discussed in this review article in some detail. Notably, the major non-polar lipid biosynthetic and hydrolytic enzymes were identified and studied in *Saccharomyces cerevisiae* at the molecular level (Table 1 and Table 2). Using the yeast system it was also possible to get a first insight into genetic and physiological links between enzymatic steps, biosynthetic and degradation pathways of non-polar lipids, and a number of other cellular processes.

Despite the progress that has recently been made in the field of yeast non-polar lipid research, many questions remained still unanswered. One of these questions concerns the redundancy of enzymes involved in non-polar lipid synthesis and degradation. The occurrence of proteins with overlapping function, acting as acyltransferases and hydrolases which use similar substrates and form similar products, seems puzzling. The simple answer to this question may be that enzymes existing in duplicate or triplicate are just backups for an important metabolic pathway. The more sophisticated view may be that these iso-enzymes rather function as individual players for specific cellular tasks and are subject to individual regulation. This type of cellular control may include classical regulation at the transcriptional and translational levels, posttranslational modifications of enzymes, but also special coordination at the organelle level. In addition, certain enzymes may be subject to protein-protein interaction and/or differential regulation by auxiliary proteins. Interaction of enzymes with each other within the biosynthetic or catabolic branch of non-polar lipid metabolism, but also regulatory aspects coordinating the apparently counterproductive processes of non-polar lipid synthesis and degradation need to be clarified.

Another open question which is related to the synthesis of non-polar lipids is the biogenesis of lipid particles (LP). The classical budding model of non-polar lipid droplets escaping from the ER has recently been challenged by alternative hypotheses, which include more specific protein targeting and the contribution of vesicle flux. Direct evidence or reliable experimental proof to support any of these models is difficult to obtain. Due to the fact that many cellular processes that might be relevant for LP biogenesis are well documented by yeast mutations may help to shed light on this problem. The yeast model may also help to pinpoint the role of auxiliary proteins in early steps of LP formation at the molecular level. Such studies will hopefully lead to a better understanding of the cellular network contributing to LP biogenesis. One important facet of such a network may be organelle communication between LP and other subcellular fractions by membrane contact or through regulatory means which are involved in the dynamic process of non-polar lipid storage and mobilization.

Finally, detailed studies of functional and structural properties of enzymes involved in non-polar lipid metabolism are still missing. A big step forward would be obtaining 3D structures of non-polar lipid metabolic enzymes. However, the hydrophobic nature of all these proteins will be a serious challenge for such studies. Structural information will also be the key to understand the enzyme-substrate interaction, the deposition of non-polar lipids in newly formed storage particles, and the accessibility of hydrolytic enzymes to specific substrates. Membrane topology studies of enzymes addressing the orientation of the respective polypeptides within their membrane environment will complete the understanding of enzyme properties, their interaction with substrates and the cell biology of non-polar lipid biosynthesis, storage and degradation in the yeast.

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Tables

Table 1

Genes and corresponding gene products involved in the metabolism of triacylglycerols in the yeast *Saccharomyces cerevisiae*

Gene	Substrate	Products	Molecular function	Subcellular localization	Synthetic lethal interaction
ARE1	Sterol (DAG)	STE (TAG)	Acyl-CoA:sterol acyltransferase, (acyl-CoA:DAG acyltransferase)	ER	*are2 + arv1*
ARE2	Sterol (DAG)	STE (TAG)	Acyl-CoA:sterol acyltransferase, (acyl-CoA:DAG acyltransferase)	ER	*are1 + arv1*
AYR1	1-acyl-DHAP	1-acyl-G-3-P	1-acyl-DHAP reductase	ER, LP	
DGA1	DAG	TAG	Acyl-CoA:DAG acyltransferase	ER, LP	
DPP1	DGPP (PtdOH)	DAG + P_i	DAG pyrophosphate phosphatase, (phosphatidate phosphatase)	Vac, Golgi, ER, membrane	
GAT1 (*GPT2*)	G-3-P DHAP	1-acyl-G-3-P 1-acyl-DHAP	G-3-P acyltransferase DHAP acyltransferase	ER, LP	*gat2*
GAT2 (*SCT1*)	G-3-P DHAP	1-acyl-G-3-P 1-acyl-DHAP	G-3-P acyltransferase DHAP acyltransferase	ER	*gat1*
LPP1	DGPP (PtdOH)	DAG + P_i	Lipid phosphate phosphatase, (phosphatidate phosphatase)	Vac, Golgi	
LRO1	DAG	TAG	Phospholipid:DAG acyltransferase	ER	
PAH1	PA	DAG + P_i	Phosphatidate phosphatase / Specific transcriptional repressor activity	Cyt, membrane	*bre1*, *slx5*, *lge1*, *ccs1*, *rad6*, *tsa1*
SLC1	Lyso-PtdOH	PtdOH	1-acyl G-3-P acyltransferase	ER, LP	*slc4***
*SLC4***	Lyso-PtdOH (Lyso-PL)	PtdOH / (PL)	1-acyl G-3-P acyltransferase, (lyso-phospholipid acyltransferase)	ER	*slc1*
TGL3	TAG	DAG + FFA	TAG lipase	LP	*gas1*, (*tgl4)**
TGL4	TAG	DAG + FFA	TAG lipase	LP, ER	*(tgl3)**
TGL5	TAG	DAG + FFA	TAG lipase	LP	

Cyt, cytosol, DAG, diacylglycerol; DGPP, DAG pyrophosphate; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum, FFA, free fatty acids; G-3-P, glycerol-3-phosphate; LP, lipid particle; PtdOH, phosphatidic acid; TAG, triacylglycerol; Vac, vacuole. Bioinformatic information was obtained from Biobase (https://www.proteome.com/tools/proteome/databases.jsp) and *Saccharomyces* Genome Database (http://www.yeastgenome.org). *synthetic growth defect; **Slc4p is also known as Ale1p and Lpt1p.

Table 2

Genes and corresponding gene products involved in the metabolism of steryl esters of the yeast *Saccharomyces cerevisiae.*

Gene	Substrate	Products	Molecular function	Subcellular localization	Synthetic lethal interaction
ARE1	Sterol (DAG)	STE (TAG)	Acyl-CoA:sterol acyltransferase, (acyl-CoA:DAG acyltransferase)	ER	*are2 + arv1*
ARE2	Sterol (DAG)	STE (TAG)	Acyl-CoA:sterol acyltransferase, (acyl-CoA:DAG acyltransferase)	ER	*are1 + arv1*
TGL1	STE	Sterol + FFA	Steryl ester hydrolase	LP	
YEH1	STE	Sterol + FFA	Steryl ester hydrolase	LP	
YEH2	STE	Sterol + FFA	Steryl ester hydrolase	PM	

DAG, diacylglycerol; ER, endoplasmic reticulum; FFA, free fatty acids; LP, lipid particle; PM, plasma membrane; STE, steryl esters; TAG, triacylglycerol.

Figures

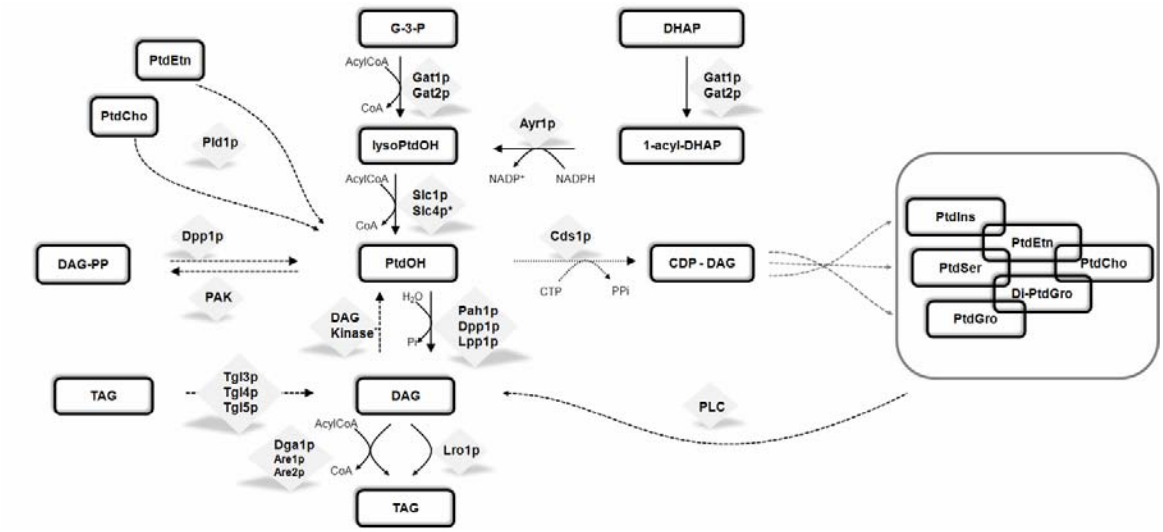

Figure 1

Biosynthesis of triacylglycerols (TAG) in *S. cerevisiae*.

Major pathways of TAG formation, enzymes involved and products formed in *S. cerevisiae* are shown. The framed part on the right documents schematically the pool of cellular phospholipids. Substrates: 1-acyl-DHAP, 1-acyl-dihydroxyacetone phosphate; DAG, diacylglycerol; DAG-PP, diacylglycerol pyrophosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; TAG, triacylglycerol. Enzymes: Are1p and Are2p, acyl-CoA:sterol acyltransferases (acyl-CoA:DAG acyltransferases); Ayr1p, 1-acyl-DHAP reductase; Cds1p, CDP–DAG synthase; Dga1p, acyl-CoA:DAG acyltransferase; Dpp1p, DAG pyrophosphate phosphatase; Gat1p and Gat2p, G-3-P acyltransferases and DHAP acyltransferases; Lpp1p, lipid phosphate phosphatase; Lro1p, phospholipid:DAG acyltransferase; Pah1p, phosphatidate phosphatase; PAK, PtdOH kinase; PLC, phospholipase C; Pld1p, phospholipase D; Slc1p and Slc4p*, 1-acyl G-3-P acyltransferases; Tgl3p, Tgl4p and Tgl5p, TAG lipases.

* Slc4p is also known as Ale1p and Lpt1p; **DAG kinase has not been identified at the molecular level in the yeast.

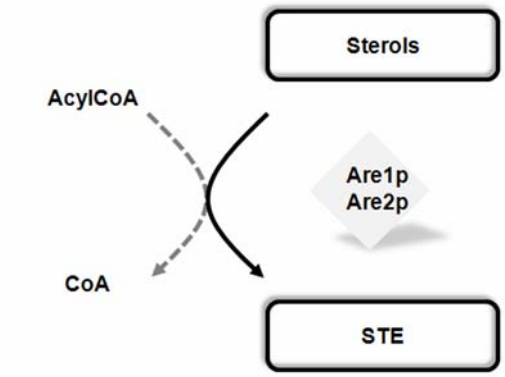

Figure 2

Biosynthesis of steryl esters (STE) in *S. cerevisiae*.

Acylation of STE in S. cerevisiae occurs in an acyl-CoA dependent reaction catalyzed by the acyl-CoA:sterol acyltransferases Are1p and Are2p. STE, steryl esters.

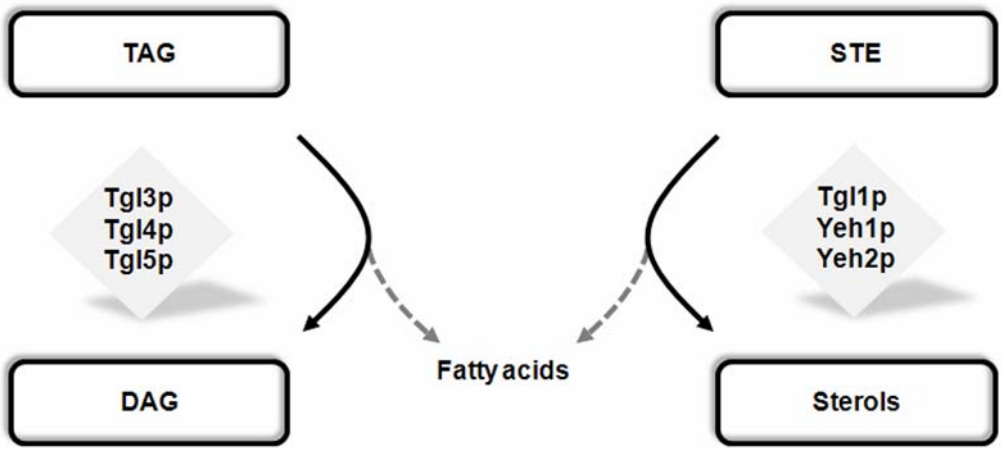

Figure 3

Hydrolysis of triacylglycerols (TAG) and steryl esters (STE) in *S. cerevisiae*.

DAG, diacylglycerol; STE, steryl esters; TAG, triacylglycerols. Enzymes: Tgl3p, Tgl4p and Tgl5p, TAG lipases; Tgl1p, Yeh1p and Yeh2p, STE hydrolases.

Chapter 2

Mobilization of steryl esters from lipid particles of the yeast

Saccharomyces cerevisiae

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Key words: sterol, steryl esters, lipid particles, lipid droplets, yeast, steryl ester hydrolase

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Abbreviations: TAG: triacylglycerols; SE: steryl esters; LP: lipid particle

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Abstract

In the yeast as in other eukaryotes, formation and hydrolysis of steryl esters (SE) are processes linked to lipid storage. In *Saccharomyces cerevisiae*, the three SE hydrolases Tgl1p, Yeh1p and Yeh2p contribute to SE mobilization from their site of storage, the lipid particles/droplets. Here, we provide evidence for enzymatic and cellular properties of these three hydrolytic enzymes. Using the respective single, double and triple deletion mutants and strains overexpressing the three enzymes, we demonstrate that each SE hydrolase exhibits certain substrate specificity. Interestingly, disturbance in SE mobilization also affects sterol biosynthesis in a type of feedback regulation. Sterol intermediates stored in SE and set free by SE hydrolases are recycled to the sterol biosynthetic pathway and converted to the final product, ergosterol. This recycling implies that the vast majority of sterol precursors are transported from lipid particles to the endoplasmic reticulum, where sterol biosynthesis is completed. Ergosterol formed through this route is then supplied to its subcellular destinations, especially the plasma membrane. Only a minor amount of sterol precursors are randomly distributed within the cell after cleavage from SE. Conclusively, SE storage and mobilization although being dispensable for yeast viability contribute markedly to sterol homeostasis and distribution.

Introduction

In all types of cells, neutral (non-polar) lipids are stored as energy reserve and source of building blocks needed for membrane formation. This is also true for the yeast *Saccharomyces cerevisiae* which synthesizes triacylglycerols (TAG) and steryl esters (SE) as the most prominent storage lipids (Czabany *et al.*, 2007). Because TAG and SE are unable to integrate into phospholipid bilayers they cluster and form the hydrophobic core of so-called lipid particles/droplets (LP). Consequently, formation of LP is tightly linked to the synthesis of TAG and SE, which occurs mainly in the endoplasmic reticulum (ER). The structure of LP is reminiscent of lipoproteins in mammals, consisting of a hydrophobic core of neutral lipids surrounded by a phospholipid monolayer with proteins embedded (Zweytick *et al.*, 2000a; Czabany *et al.*, 2008).

Sterols are essential lipid components and serve as constituents of membranes to maintain membrane permeability and fluidity. Moreover, sterols affect the aerobic metabolism (Smith and Parks, 1993; Smith and Parks, 1997), the cell cycle (Dahl *et al.*, 1987) as well as sterol uptake and sterol transport (Lorenz *et al.*, 1986; Tuller *et al.*, 1999). Sterols of eukaryotes exist as two major forms, namely as free sterols and steryl esters (SE). Beside other regulatory mechanisms affecting sterol biosynthesis, esterification of ergosterol and sterol intermediates, storage of SE in LP, and hydrolysis of SE contribute to cellular sterol homeostasis in the yeast. These processes do not only allow cells to conserve chemical energy, which can be used in times of deprivation, but also provide important means to balance subcellular levels of free sterols and fatty acids, which are essential and critical for cell structure and function.

In the yeast *Saccharomyces cerevisiae*, sterols are esterified in the ER by two enzymes with overlapping acyl-CoA:sterol acyltransferase activities, Are1p and Are2p (Zinser *et al.*,

1993; Yang *et al.*, 1996; Yu *et al.*, 1996; Zweytick *et al.*, 2000b). These two enzymes are 49% identical to each other and exhibit approximately 24% identity to human acyl-CoA:cholesterol acyltransferase (ACAT). Are2p is the major yeast SE synthase with a preference to esterify ergosterol, whereas a significant preference for sterol precursors, mainly lanosterol, was detected for Are1p (Yang *et al.*, 1996; Zweytick *et al.*, 2000b). Moreover, it was reported that Are1p is the main contributor to SE synthesis under anaerobic conditions (Valachovic *et al.*, 2002).

The highest yeast SE hydrolase activity was detected in the plasma membrane (Zinser *et al.*, 1993). Recently, the SE hydrolase Yeh2p which catalyzes this reaction was identified as the first yeast enzyme of this kind at the molecular level (Köffel *et al.*, 2005; Müllner *et al.*, 2005). Yeh2p is homologous to several known mammalian SE hydrolases (Miled *et al.*, 2000). Since in a *yeh2Δ* deletion strain bulk SE mobilization occurred at a similar rate as in wild type, it was assumed that Yeh2p was not the only SE hydrolase, but additional enzymes with overlapping function have to exist. Indeed, a bioinformatic approach identified two more members of the SE hydrolase family recently in *Saccharomyces cerevisiae* (Köffel *et al.*, 2005; Jandrositz *et al.*, 2005). These enzymes encoded by *YEH1* and *TGL1* are, similar to Yeh2p, paralogues of the mammalian acid lipase family. In contrast to Yeh2p, Yeh1p and Tgl1p are LP proteins. The two LP SE hydrolases exhibit a very low activity *in vitro*, but a triple deletion in combination with *YEH2* abolished SE hydrolysis completely. Yeh1p was shown to be the most important SE hydrolase in a *hem1Δ* deletion strain mimicking anaerobic growth, whereas Yeh2p and Tgl1p were largely inactive under these conditions (Köffel and Schneiter, 2006).

In the study presented here, we used a biochemical approach employing terbinafine, an inhibitor of yeast sterol synthesis, to screen a number of further candidate gene products for their possible role as SE hydrolases and to investigate at the same time enzymatic properties

of Tgl1p, Yeh1p and Yeh2p in some more detail. This screening did not identify additional mutants bearing defects in SE hydrolysis. However, our experiments demonstrated that Tgl1p, Yeh1p and Yeh2p exhibit a certain degree of specificity resulting in changes in the total and esterified sterol fractions of respective mutant strains. We also show that SE turnover recycles sterol intermediates to the sterol biosynthetic pathway and thus contributes to a balanced cellular level of free ergosterol. Links between SE synthesis, hydrolysis and sterol biosynthetic flux are discussed.

Material and Methods

Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. The wild type strain *Saccharomyces cerevisiae* BY4742 and strains bearing single deletions of nonessential genes were obtained from EUROSCARF (Winzeler *et al.*, 1999). Cells were grown in rich medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD) or 2% galactose (YPGal), or in minimal medium containing 0.67% yeast nitrogen base and 2% glucose (MMGlu) or 2% galactose (MMGal) supplemented with the appropriate amino acids. Double and triple mutant strains were generated by crossing of single mutants using standard procedures for *Saccharomyces cerevisiae*.

YEH1 and *TGL1* were tagged in the genome of wild type BY4742 (Table 1) by linear transformation with PCR products from the template plasmids of pFA6a-kanMX6-PGAL1 (Longtine *et al.*, 1998) (insertion of a gal-promotor) and YCplac33-ZZ (Whyte and Munro, 2001) (insertion of a C-terminal ProteinA-tag to the full length protein). In the first transformation step, the gal-promotor was inserted, and in a second step the PCR cassette for the C-terminal ProteinA-tag was transformed into the strains harboring the gal-promotor.

Primers used in this study are listed in Table 2. The different constructs and the respective recombinant strains used in this study are referred to as *TGL1*-protA and *YEH1*-protA. The correctness of insertions was tested by resistance of strains to geneticin. Moreover, identity of all strains was confirmed by colony PCR and DNA sequencing.

Subcellular fractionation of yeast cells

Subcellular fractions of yeast cells were prepared by published procedures (Serrano, 1988; Leber *et al.*, 1994; Zinser and Daum, 1995). Protein was quantified by the method of Lowry *et al*. (Lowry *et al.*, 1951) using bovine serum albumin as standard. Prior to protein analysis, samples of LP were delipidated. Non-polar lipids were extracted by two volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were analyzed as described above. Subcellular fractions were routinely tested by Western blot analysis (Haid and Suissa, 1983) using rabbit antibodies against Erg1p, Por1p, Wbp1p and Prc1p/CPY. Overexpression of Protein A tagged SE hydrolases was tested with an antibody against Protein A (Sigma, 1:30.000). Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Lipid analysis

Lipids from yeast cells grown on YPD or MMGal were extracted as described by Folch *et al*. (Folch *et al.*, 1957). For quantification of neutral lipids extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4), and chromatograms were developed in an ascending manner by using the solvent system light petroleum/diethyl ether/acetic acid (25:25:1; per vol.) for the first third of the distance.

Then, plates were briefly dried and further developed to the top of the plate using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of ergosterol and ergosteryl esters was carried out by densitometric scanning at 275 nm with a Shimadzu dual wavelength chromatoscanner CS-930 using ergosterol as standard.

For gas liquid chromatography/mass spectrometry (GLC/MS) of sterols (Quail and Kelly, 1996) either whole cells (corresponding to 20 ml culture with an OD_{600} of 1), 300 µg of homogenate protein, 300 µg of plasma membrane protein or 30 µg of LP protein, respectively, were incubated for 2h at 90°C together with 0.6 ml of methanol, 0.4 ml of 0.5% pyrogallol dissolved in methanol, 0.4 ml of 60% aqueous KOH and 10 µg of cholesterol dissolved in ethanol as an internal standard. Lipids were extracted three times with *n*-heptane and combined extracts were taken to dryness under a stream of nitrogen. Then, lipids were dissolved in 10 µl of pyridine, and after adding 10 µl of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (Sigma) samples were diluted with ethyl acetate to an appropriate concentration. GLC/MS analysis of silylated sterol adducts was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA), equipped with an HP 5972 mass selective detector, and HP 5-MS column (cross-linked 5% phenyl methyl siloxane; dimensions 30 m x 0.25 mm x 0.25 µm film thickness). Aliquots of 1 µl were injected in the splitless mode at 270°C injection temperature with helium as a carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 310°C. Mass spectra were acquired in scan mode (scan range 200-259 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

Mobilization of steryl esters in vivo

Mobilization of SE *in vivo* was tested as described by Leber *et al*. (Leber *et al.*, 1995). In brief, yeast cells were pregrown in YPD medium for 16 h and transferred to sterol-free medium (MMGluc). Terbinafine dissolved in ethanol was added at a final concentration of 30 µg/ml. The optical density was measured with a Hitachi U-1100 UV/VIS Spectrophotometer at a wavelength of 600 nm, and aliquots of the cultures were withdrawn at different time points. Cells were harvested by centrifugation, washed once with H_2O, and used for lipid analysis as described above.

Enzymatic analysis

The enzymatic assay measuring acyl-CoA:ergosterol acyltransferase activity was performed in a final volume of 100 µl containing 6 nmol of [^{14}C]oleoyl-CoA (88,000 dpm), 0.025 mM ergosterol, 0.5 mM CHAPS, 100 mM KH_2PO_4 (pH 7,4), 1 mM dithiothreitol and 200 µg protein of yeast homogenate from cells grown to logarithmic phase (Yang *et al.*, 1997). Incubations were carried out for 30 min at 30°C and terminated by addition of 300 µl chloroform:methanol (2:1, v/v). Lipids were extracted for 10 min with shaking at room temperature and the extraction was repeated once under identical conditions. Organic phases were combined and washed twice using methanol:water:chloroform (47:48:3, per vol.). The organic phase was taken to dryness under a stream of nitrogen. Lipids were dissolved in 30 µl chloroform:methanol (2:1; v/v), separated by TLC as described above and visualized on TLC plates by staining with iodine vapor. Bands were scraped off, and radioactivity was measured by liquid scintillation counting using LSC Safety Cocktail (Baker, Deventer, The Netherlands) and 5% water as a scintillation mixture.

Results

Steryl ester hydrolase activity of Tgl1p and Yeh1p in vivo

Recently, the three major steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p of the yeast *Saccharomyces cerevisiae* were identified by different strategies in different laboratories (Köffel *et al.*, 2005; Müllner *et al.*, 2005; Jandrositz *et al.*, 2005). As an extension to previous studies in our laboratory (Müllner *et al.*, 2005) we performed an *in vivo* screening to identify possible additional proteins involved in yeast SE hydrolysis, and to obtain at the same time more biochemical information about the less characterized gene products of *TGL1* and *YEH1*. For this purpose, we analyzed 25 candidate mutant strains whose gene products had sequence homologies to previously identified hydrolytic enzymes and/or a predicted localization in LP (Table 3).

When these candidate strains were cultivated on glucose containing medium in the presence of terbinafine, an inhibitor of sterol biosynthesis, sterols stored in the form of SE in LP were hydrolyzed to yield the balanced level of free sterols required for membrane formation (Leber *et al.*, 1995). Samples were withdrawn after different time points, lipids were extracted and the relative content of ergosteryl esters was analyzed after TLC separation of neutral lipids. These experiments showed that SE from wild type were mobilized within 4 hours (Fig. 1). Strains deleted of *YEH1* and *TGL1* exhibited a slightly decreased rate of SE hydrolysis confirming the contribution of these gene products to neutral lipid mobilization in the living yeast cell. Similar results using *yeh1Δ* and *tgl1Δ* mutants had been reported before by Köffel *et al*. (Köffel *et al.*, 2005). Surprisingly, Müllner *et al*. (Müllner *et al.*, 2005) and Köffel *et al*. (Köffel *et al.*, 2005) had shown that under the same inhibitory conditions deletion of *YEH2* did not lead to a decreased rate of SE mobilization. Overexpression of Tgl1p, Yeh1p or Yeh2p, respectively (Fig. 2), did not significantly enhance SE hydrolysis

(data not shown). This result may be due to the fact that rather supply of the substrate(s) than the activity of the enzyme(s) is limiting for the mobilization of SE. Other deletion strains tested (see Table 1), which were also subjected to SE mobilization assays, were not affected in their capacity to hydrolyze SE (data not shown). Thus, it appears that the three known SE hydrolases are the major or probably the only relevant enzymes of this kind from the collection of candidate gene products tested.

Steryl ester profile of strains lacking steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p

Results obtained during the screening procedure described above led us to study (i) cellular consequences of deletions of genes encoding the three known SE hydrolases, and (ii) enzymatic properties of the respective enzymes with emphasis on the substrate specificity. The fact that *in vivo* SE from *tgl1Δ* and *yeh1Δ* mutants were not mobilized as efficiently as in wild type led us to investigate the cellular level of SE in these mutants. Quantification of SE levels from the three single knockout strains and the triple knockout strain *yeh1Δyeh2Δtgl1Δ* revealed that in the triple knockout strain and in the *tgl1Δ* deletion strain a marked accumulation of SE over wild type occurred (Fig. 3). This effect was not observed with *yeh1Δ* and *yeh2Δ* deletion strains.

Whole cell sterol analysis from *tgl1Δ*, *yeh1Δ* and *yeh2Δ* deletion strains showed that single gene deletions had only minor effects on the total cellular sterol composition (Table 4). The slight changes of values with *tgl1Δ* and *yeh2Δ* are of borderline significance. In all three deletion mutants, however, the amount of total cellular sterol was slightly increased. When *Yeh1*-protein A, *Yeh2*-protein A or *Tgl1*-protein A hybrids were overexpressed, the level of total cellular ergosterol was slightly decreased compared to wild type, whereas amounts of sterol precursors were markedly reduced. The only exception with that respect was a strain overexpressing a *Tgl1*-protein A fusion protein exhibiting a slightly higher lanosterol level

than wild type. Most importantly, however, the amount of total cellular sterols was significantly decreased in the overexpressing strains. It has to be noted, that values of whole cell analysis include amounts of free sterols and SE. This result indicates that changes in SE mobilization affect total sterol homeostasis. Regulatory aspects that may cause the observed changes will be discussed below.

Quantitative analysis of individual SE from wild type and mutant strains overexpressing individual SE hydrolases revealed more specific differences in the SE pattern. As shown before by Müllner *et al*. (Müllner *et al.*, 2005), Yeh2p has a preference for SE containing ergosterol, fecosterol and especially zymosterol as a sterol moiety (Fig. 4). Similar results were obtained with a strain overexpressing a *Yeh1*-protein A hybrid. Results with a transformant overexpressing *Tgl1*-protein A were different insofar as in this strain mobilization of SE was restricted to ergosteryl and zymosteryl derivatives. Most interestingly, in none of the strains overexpressing SE hydrolases the level of lanosteryl esters was reduced. These results are in line with data shown in Table 4. In summary, the three different SE hydrolases exhibit different, although not exclusive substrate specificities.

Sterol precursors from steryl esters are efficiently converted to ergosterol

Ergosterol set free from ergosteryl esters of LP can be directly utilized as a component for membrane biogenesis. In contrast, precursor sterols are only incorporated into membranes under specific conditions, e.g., when ergosterol synthesis is blocked by mutations. As an example, in an *erg11Δerg3Δ* mutant lanosterol, 4,14-dimethylcholesta-8,24-dienol and 14-methylfecosterol had been shown to be readily utilized as plasma membrane components (Ott *et al.*, 2005). Here, we demonstrate that wild type cells when poisoned with terbinafine, which created a bottleneck in the formation of ergosterol, efficiently converted sterol precursors to ergosterol within a few hours (Fig. 5). Since SE are the major source of these sterol

precursors, SE hydrolysis is prerequisite for metabolic conversion of these precursors to ergosterol. The availability of single, double and triple mutants bearing deletions of SE hydrolases provided a possibility to test the contribution of individual enzymes to this mobilization process. To address this question, we performed experiments *in vivo* with the different mutant strains in the presence of the ergosterol biosynthesis inhibitor terbinafine.

In *yeh1*Δ*tgl1*Δ and *yeh1*Δ*yeh2*Δ double mutants, which contain only active Yeh2p or Tgl1p, respectively, sterol precursor conversion to ergosterol was only slightly reduced compared to wild type which paralleled the moderately reduced SE hydrolase activity in these strains. Concomitantly with the decrease of sterol precursors the amount of ergosterol increased in these strains within 6 hours treatment with terbinafine. In the *yeh2*Δ*tgl1*Δ double deletion strain with Yeh1p as the only active SE hydrolase, precursor sterols from SE were also efficiently utilized, although the level of ergosterol was not increased in parallel after terbinafine treatment. In a *yeh1Δyeh2Δtgl1Δ* triple mutant, precursor sterols rather accumulated in SE and ergosterol did not increase after terbinafine treatment, because ergosterol synthesis was inhibited and sterol precursors stored in the form of SE could not be utilized for conversion to ergosterol. Without terbinafine, an increased level of ergosterol and precursors stored in SE was detected, indicating that ergosterol synthesis was ongoing while the SE mobilization was completely blocked.

The fact that the synthesis of ergosterol was inhibited upon terbinafine treatment of cells, but sterol precursors from SE were mobilized to fill the gap (see Fig. 5), avoided lack of ergosterol during the observed time period. Consequently, the membrane with the highest requirement for ergosterol, the plasma membrane, was not depleted of this lipid within this time range (Table 5). In wild type cells, the plasma membrane contained ergosterol as the only sterol component after treatment with terbinafine for 6 hours due to the fact that all precursor sterols were efficiently recycled (see Fig. 5). This result also indicated that

precursor sterols were channeled to their site of further conversion to ergosterol, the ER, and not randomly distributed among cellular membranes. In the plasma membrane of the *yeh1Δyeh2ΔtglΔ* triple mutant the level of ergosterol remained high during the treatment with terbinafine.

Interestingly, a small but significant amount of lanosterol was detected in the plasma membrane of the triple mutant. The failure of this strain to turnover sterol precursors through the SE storage route may be the reason of the observed effect. The slight accumulation of unesterified lanosterol as result of a feedback regulation caused by the lack of SE hydrolases may have led to a random distribution of this sterol as had been shown before with an *erg11Δerg3Δ* mutant (Ott *et al.*, 2005).

Another question that evolved from this scenario was whether a strain which was not able to hydrolyze SE, had a reduced capacity to synthesize SE in a kind of feedback regulation. Thus, SE synthase activity was measured in a *yeh1Δyeh2ΔtglΔ* triple mutant and in wild type. *In vitro* assays showed that the activity of Are1p and Are2p was slightly reduced in the *yeh1Δyeh2ΔtglΔ* triple mutant. In wild type homogenate, SE synthase activity of 9.8 pmol/min/mg protein was measured compared to an activity of 6.8 pmol/min/mg protein in the triple deletion strain.

Discussion

Results presented in this study confirm that the three SE hydrolases, Tgl1p, Yeh1p and Yeh2p, are most likely the only enzymes of this kind in the yeast *Saccharomyces cerevisiae*. Considering the different functions of sterols in the cell, it is of major importance that sterol homeostasis including sterol biosynthesis, storage and utilization is strictly regulated. This

regulation also concerns SE cleavage as an important metabolic branch to maintain a balance between free and esterified sterols. Moreover, a flexible SE pool may help to defend the cell against both a surplus and lack of free sterols. This scenario leaves us with the question, why *Saccharomyces cerevisiae* contains three SE hydrolyzing enzymes with different subcellular localization and at least slightly different specificities. It has to be noted, however, that none of these enzymes alone or in combination with the other SE hydrolases is essential for survival of the yeast at least under standard growth conditions.

An interesting biochemical property of the three different yeast SE hydrolases is their specificity for substrates. It has been demonstrated before that Yeh2p exhibited a slight preference for zymosteryl esters as a substrate (Müllner *et al.*, 2005). Here, we show that Tgl1p has a similar preference for zymosteryl esters of LP although ergosteryl esters were also properly utilized. Yeh1p also cleaved ergosteryl and zymosterol esters, but utilized fecosteryl esters more efficient than the other two SE hydrolases. Interestingly, none of the SE hydrolases used lanosteryl esters as a preferred substrate under the conditions tested. In contrast, it had been shown before, that under anaerobic or heme-deficient conditions Yeh1p is equally active against cholesteryl, lanosteryl and ergosteryl esters (Köffel and Schneiter, 2006). Thus, SE hydrolases exhibit distinct although not exclusive substrate specificities.

Sterol precursors set free from SE are not directly incorporated into the plasma membrane. Analysis of the plasma membrane from wild type cells treated with terbinafine revealed that sterol precursors were not detectable at substantial amounts in this compartment, but the level of ergosterol was maintained. Thus, blocking ergosterol biosynthesis led to mobilization of sterol precursors stored in SE and transport of these intermediate sterols to the ER where conversion to ergosterol occurred. Ergosterol synthesized from precursor sterols was then translocated and incorporated into cellular membranes, e.g., the plasma membrane. In a triple deletion strain lacking all three major SE hydrolases, sterol precursors stored as

moieties of SE could not be utilized. Thus, the amount of ergosterol in the plasma membrane was held constant (see Table 5). Interestingly, however, lanosterol was detected in the plasma membrane of this strain which was not observed for wild type. We believe that a feedback regulation to sterol precursor usage may be the reason for this observation. Lanosterol appears to be one of the sterol precursors that can move rather freely between organelles as demonstrated in a study using *erg11*Δ*erg3*Δ double mutants (Ott *et al.*, 2005). In this work it had been shown that lanosterol did neither accumulate at its side of synthesis, the LP, nor at its site of further conversion, the ER, but was more or less randomly distributed among all organelles. This non-directed migration of lanosterol is in contrast to ergosterol traffic which seems to occur by more distinct transport mechanisms (Zinser *et al.*, 1993; Baumann *et al.*, 2005; Schnabl *et al.*, 2005; Sullivan *et al.*, 2006). Mechanisms of ergosterol transport to the plasma membrane under discussion are vesicle flux or membrane contact. Moreover, Arv1p which had been identified as a mediator of eukaryotic sterol homeostasis seems to play a role in sterol migration to the plasma membrane (Tinkelenberg *et al.*, 2000), and also Osh proteins (oxysterol binding protein homologues) showed an effect on the delivery of newly synthesized ergosterol from ER to the plasma membrane (Schulz and Prinz, 2007).

In summary, data presented here demonstrate that turnover of SE is of similar importance for sterol homeostasis as sterol esterification. Yang *et al*. (Yang *et al.*, 1996) showed that the inability to esterify intracellular sterols down regulated the sterol biosynthesis thus maintaining the critical low level of free sterols. Mechanisms of this feedback regulation to sterol synthesis by the SE pool in yeast are poorly understood. Data presented here suggest that similar mechanisms may be relevant for cells which are not able to hydrolyze SE and set sterols free from these storage lipids.

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Tables

Table 1:

S. cerevisiae strains used in this study

Strain	Relevant genotype	Source and/or reference
BY4742	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1*	EUROSCARF
YLL012w	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh1::kanMX4*	EUROSCARF
YKL140w	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 tgl1::kanMX4*	EUROSCARF
YLR020c	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh2::kanMX4*	EUROSCARF
YAW1	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh1::kanMX4*	This study
YAW2	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh1::kanMX4 yeh2:*	This study
YAW3	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh2::kanMX4*	This study
YAW4	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 yeh1::kanMX4 yeh2::kanMX4 tgl1::kanMX4*	This study
YAW5	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 kanMX6-PGAL1-YEH1-*	This study
YAW6	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 kanMX6-PGAL1-TGL1-*	This study
YEH2-	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 kanMX6-PGAL1-YEH2-*	Müllner *et al.*,
HIS-*YEH2*	*Matα lys2Δ0 leu2Δ0 ura3Δ0 his3Δ1 kanMX6-PGAL1-His$_6$-*	Müllner *et al.*,

Table 2:

Primers used for *YEH1* and *TGL1* constructs

	Sequence 5´→ 3´
gal-prom *YEH1* (fw)	GATAAAGTAATAGTTTTATATATAGGTATATTTACTGCACAAT TCACACGGAATTCGAGCTCGTTTAAAC
gal-prom *YEH1* (rev)	GAACGTTGCTAGTAAATTCCTAGCTCTTTTCAACACCGCAGA AACACCCATTTTGAGATCCGGGTTTT
gal-prom *TGL1* (fw)	ACAAAACTTTATTATTCTAGCACTATTTTAAAAAACTGTCTTT TGGCAAAGAATTCGAGCTCGTTTAAAC
gal-prom *TGL1* (rev)	GACTATAATGTAATCTGTTATCGATAATCTGCCTAAAAAGGG GAAGTACATTTTGAGATCCGGGTTTT
C-terminal protein A *YEH1*	GTTCGGACACAGAGGTGGAAACGGAGCTGGAAATGGTTGCT GAGAAGGCTGGAGCAGGGGCGGGTGC
C-terminal protein A *YEH1*	TATGTATTCCCAAGTATAATTTATATTAACCTATATATCATGC TTCCTCTCCCCTCGAGGTCGACGGTATCG
C-terminal protein A *TGL1*	CAACTAGATGCCAACTCTTCGACAACTGCGCTGGATGCTCTA AATAAAGAAGGAGCAGGGGCGGGTGC
C-terminal protein A *TGL1*	TATTATCCTAGACAAAAAATAGTTTAATAGGGTTTCTCACGC ATTCTTTCCCCTCGAGGTCGACGGTATCG

Table 3:

Genes encoding putative steryl ester hydrolases

ORF	Gene	Localization (GFP database)	Characteristics
YBR042c	*CST26*	LP	Putative acyltransferase
YBR177c	*EHT1*	LP	Alcohol acyltransferase, hydrolase activity
YBR204c		LP/ER	Serine hydrolase activity
YCL005w	*LDB16*	LP	Unknown
YCR068w	*ATG15*	?	Putative lipase
YDL193w	*NUS1*	LP/ER	Prenyltransferase activity
YDR058c	*TGL2*	?	Putative triglyceride lipase
YDR275w	*BSC2*	LP	Unknown
YDR425w	*SNX41*	LP/endosome	Involved in retrograde transport, endosome to Golgi
YGL098w	*USE1*	LP	SNARE protein of ER, retrograde transport from Golgi to ER
YJR107w		?	Lipase activity
YKL094w	*YJU3*	LP/ER	Serine hydrolase activity
YKL140w	*TGL1*	LP	STE hydrolase
YKL179c	*COY1*	LP	Member of CASP family of Golgi proteins
YKR046c	*PET10*	LP	Protein involved possibly in respiration
YLL012w	*YEH1*	LP	STE hydrolase
YMR031c		?	Unknown
YMR110c		LP	Similarity to aldehyde dehydrogenases
YMR148w		LP	Unknown
YMR152w	*YIM1*	ER/LP	unknown
YOR059c		LP	Unknown
YOR246c		LP	Protein containing a short dehydrogenase domain
YPL232w	*SSO1*	LP	SNAP receptor activity
YPR089w		?	Unknown
YDR525w-A	*SNA2*	LP	Protein involved in Prc1p, Pep4p and Pho8p trafficking to the vacuole

Table 4:

Whole cells sterol analysis from wild type (BY4742) and mutant strains bearing defects in steryl ester hydrolases
The amount of sterols from whole cell extracts in µg per OD_{600} includes free sterols and SE. Data are mean values from 5 independent experiments. All mutant strains are in BY4742 background. Strain genotypes are shown in Table 1 (n.d. = not detectable). PA, protein A.

µg sterol/OD_{600}							
	BY4742	Δ*yeh1*	Δ*yeh2*	Δ*tgl1*	*Yeh1*-PA	*Yeh2*-PA	*Tgl1*-PA
Zymosterol	3.0 ± 1.00	4.0 ± 0.03	4.4 ± 0.57	3.3 ± 1.00	0.4 ± 0.32	0.5 ± 0.15	1.5 ± 0.63
Ergosterol	15.5 ± 1.50	14.2 ± 2.00	17.0 ± 0.04	17.9 ± 1.00	14.6 ± 3.91	13.1 ± 3.73	15.1 ± 4.14
Fecosterol	2.2 ± 0.57	3.5 ± 0.57	3.0 ± 0.02	2.0 ± 0.05	n.d.	0.6 ± 0.32	0.5 ± 0.48
Lanosterol	1.7 ± 0.57	1.4 ± 0.57	2.0 ± 0.07	1.3 ± 0.03	1.5 ± 0.52	1.6 ± 0.32	2.5 ± 0.14
Total sterol	22.4	23.1	26.4	24.5	16.5	15.8	19.6

Table 5:

Sterol analysis of plasma membrane from wild type (BY4742) and the triple deletion mutant (TM) *yeh1*Δ*yeh2*Δ*tgl1*Δ after terbinafine treatment
Values are from 3 independent measurements with a mean deviation of ± 10% and expressed as µg sterol/mg protein (n.d. = not detectable). Time points 0 h (T=0h), time point 6 h without terbinafine [T=6h(-)] and time point 6 h with terbinafine treatment [T=6h(+)] are shown.

µg sterol / mg plasma membrane protein						
	BY4742 T=0h	BY4742 T=6h(-)	BY4742 T=6h(+)	TM T=0h	TM T=6h(-)	TM T=6h(+)
Zymosterol	0.83	n.d.	n.d.	n.d.	n.d.	n.d.
Ergosterol	165.65	153.02	205.60	232.71	202.56	234.96
Fecosterol	0.89	n.d.	n.d.	n.d.	n.d.	n.d.
Lanosterol	0.91	0.59	n.d.	1.64	2.85	3.7

Figures

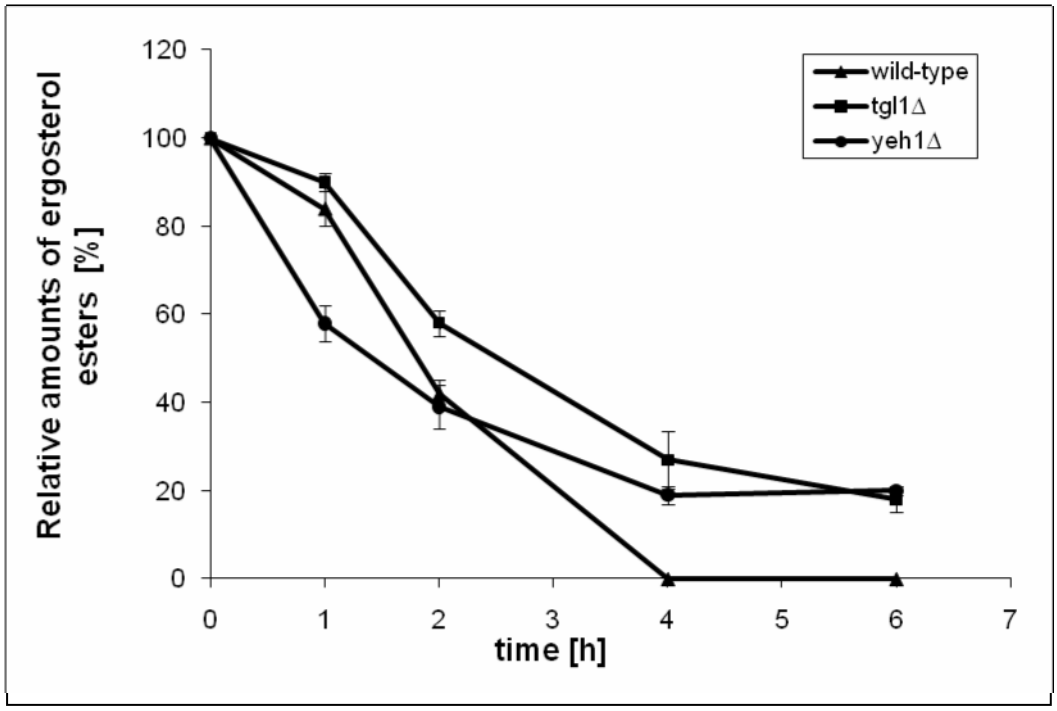

Figure 1

Lack of *YEH1* and *TGL1* affects the efficiency of steryl ester mobilization *in vivo*.

Wild type (BY4742), *yeh1*Δ and *tgl1*Δ strains were grown as described in the methods section and transferred to sterol free media. Samples were withdrawn at 0, 1, 2, 4 and 6h after incubation with terbinafine. Lipids were extracted and the amount of SE was determined after separation by TLC (see Material and Methods).

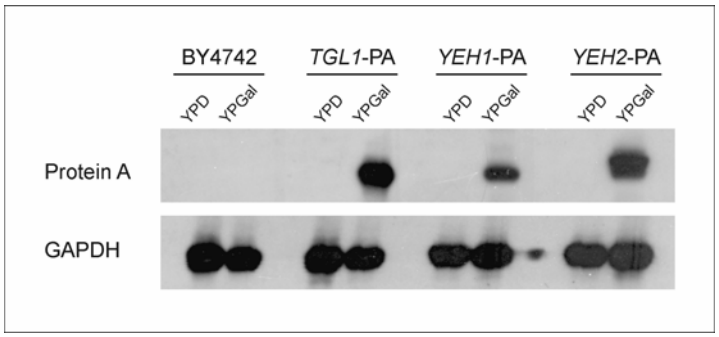

Figure 2

Expression of protein A tagged Tgl1p, Yeh1p and Yeh2p.

Protein A tagged hybrids of Tgl1p, Yeh1p and Yeh2p were constructed as described in the Methods section. Immunoblot analysis was performed after separation of proteins by SDS-PAGE using an antibody against Protein A. The wild type strain BY4742 not expressing a Protein A tagged polypeptide was used as a negative control. Non-induced (YPD) and induced conditions (YPGal) were compared. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a highly abundant cytosolic protein, was used as a loading control.

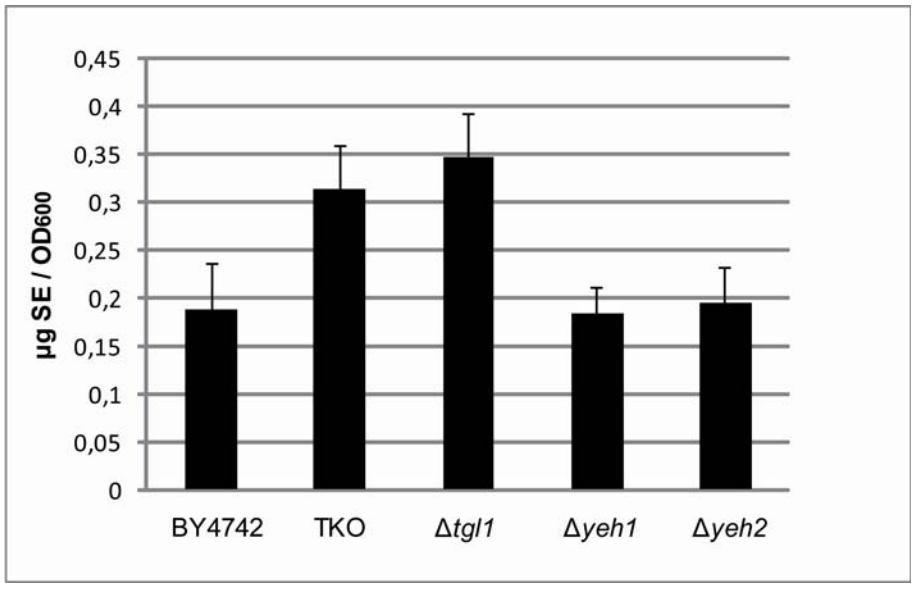

Figure 3

Cellular levels of steryl esters in strains lacking steryl ester hydrolases.

SE from wild type, the triple mutant *yeh1*Δ*yeh2*Δ*tgl1*Δ (TKO), and the single mutants *yeh1*Δ, *yeh2*Δ and *tgl1*Δ were quantified as described in the Methods section. Data are mean values from 5 independent experiments.

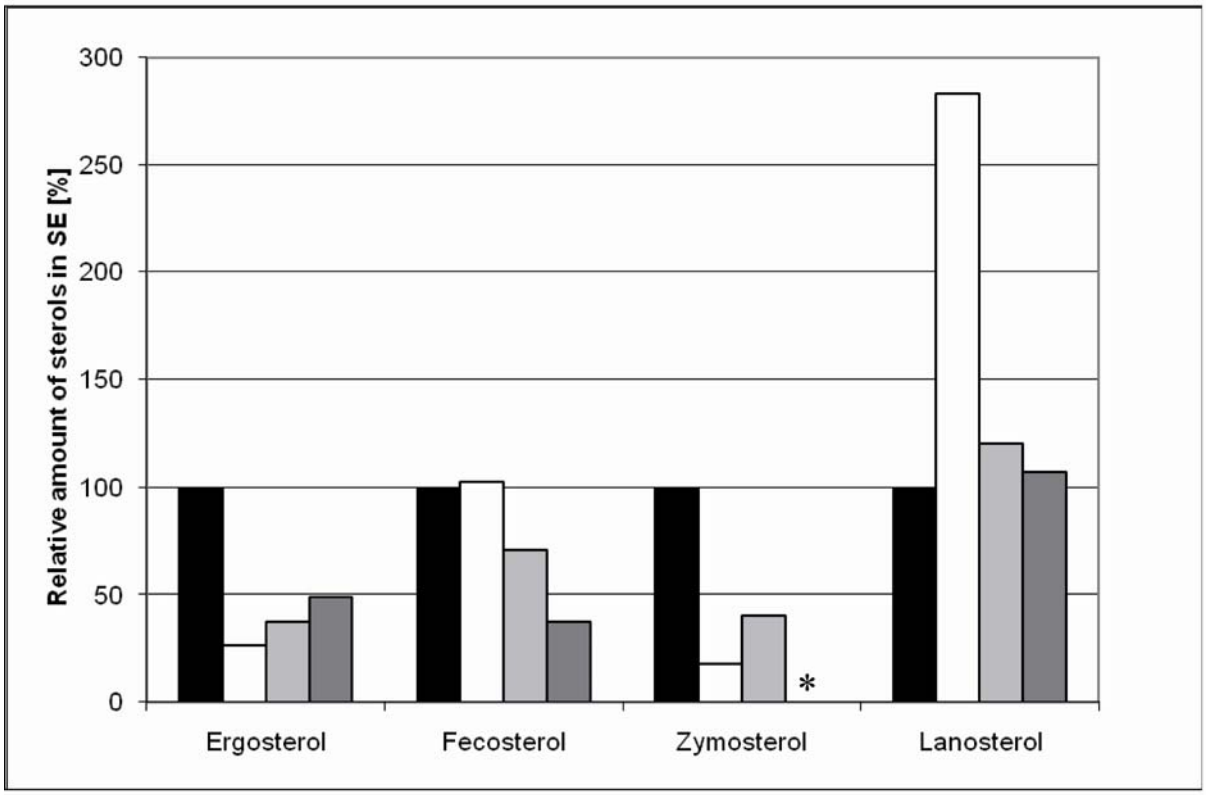

Figure 4

Substrate specificity of Yeh1p, Yeh2p and Tgl1p overexpressing strains compared to wild type BY4742.

Relative amounts of SE isolated from LP of wild type (*black bars*) and strains overexpressing *TGL1*-protA (*white bars*), *YEH1*-protA (*light grey bars*) and *YEH2*-protA (*dark grey bars*) are shown. Values obtained from wild type were set at 100 %. Data are mean values from 3 independent experiments with a MD of ± 10%. * = not detectable.

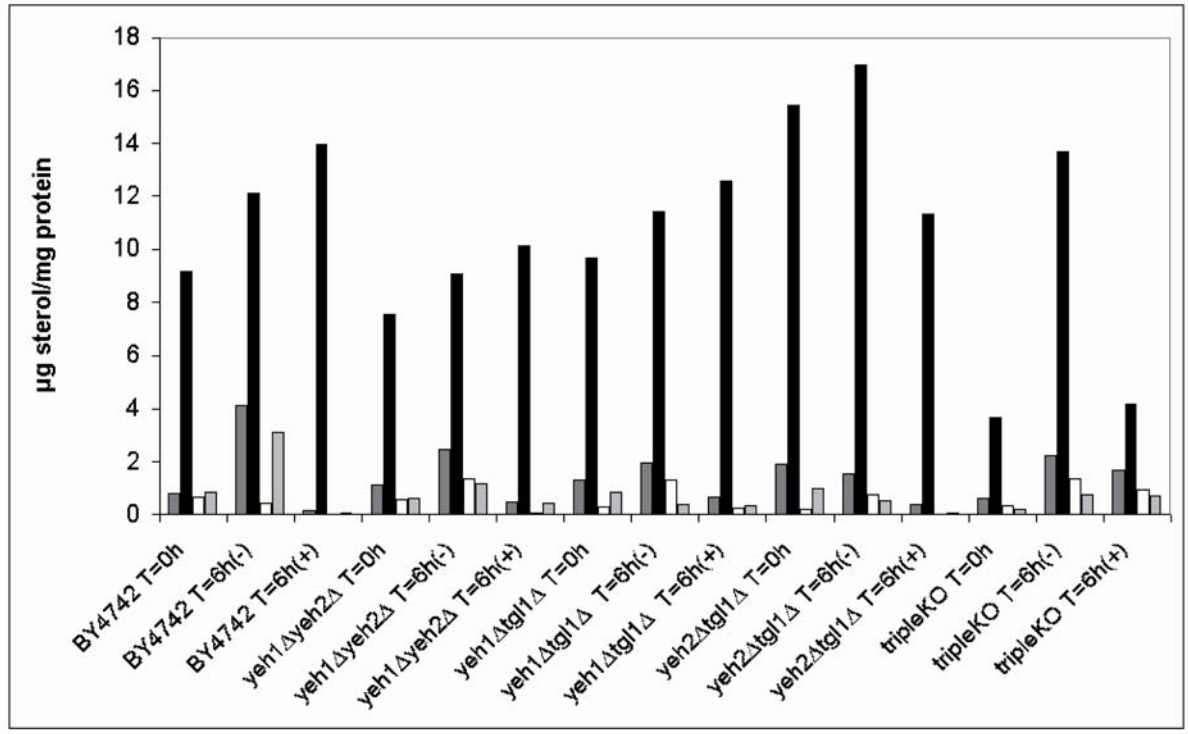

Figure 5

Channeling of sterol precursors set free from steryl esters.

Wild type BY4742 and mutant cells bearing defects in SE hydrolytic enzymes pregrown in rich medium (T=0) were incubated for 6 h in the absence (-) or presence (+) of terbinafine. Lipids were extracted and sterols were analyzed by GLC/MS as described in the Methods section. Data are mean values from three independent experiments with a maximum MD of ±10 %. Zymosterol (*dark grey bars*), ergosterol (*black bars*), fecosterol (*white bars*), lanosterol (*light grey bars*).

Chapter 3

Modulation of sterol homeostasis by the Cdc42p effectors Cla4p and Ste20p in the yeast *Saccharomyces cerevisiae*

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Keywords: cell polarity; p21-activated kinases; sterol; steryl ester; yeast

Abbreviations: GST, glutathione S-transferase; PAK, p21-activated kinase; SC, synthetic complete; SE, steryl esters; YPD, 1 % yeast extract, 2% peptone, 2% dextrose.

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Abstract

The conserved Rho-type GTPase Cdc42p is a key regulator of signal transduction and polarity in eukaryotic cells. In the yeast Saccharomyces cerevisiae, Cdc42p promotes polarized growth through the p21-activated kinases Ste20p and Cla4p. Previously, we demonstrated that Ste20p forms a complex with Erg4p, Cbr1p and Ncp1p, which all catalyze important steps in sterol biosynthesis. *CLA4* interacts genetically with *ERG4* and *NCP1*. Furthermore, Erg4p, Ncp1p and Cbr1p play important roles in cell polarization during vegetative growth, mating and filamentation. As Ste20p and Cla4p are involved in these processes it seems likely that sterol biosynthetic enzymes and p21-activated kinases act in related pathways. Here, we demonstrate that the deletion of either *STE20* or *CLA4* results in increased levels of sterols. In addition, higher concentrations of steryl esters, the storage form of sterols, were observed in *cla4Δ* cells. *CLA4* expression from a multicopy plasmid reduces enzyme activity of Are2p, the major steryl ester synthase, under aerobic conditions. Altogether, our data suggest that Ste20p and Cla4p may function as negative modulators of sterol biosynthesis. Moreover, Cla4p has a negative effect on steryl ester formation. As sterol homeostasis is crucial for cell polarization, Ste20p and Cla4p may regulate cell polarity in part through the modulation of sterol homeostasis.

Introduction

The Rho-type GTPase Cdc42p plays a crucial role in the establishment and maintenance of cell polarity (Etienne-Manneville, 2004; Jaffe and Hall, 2005). In the budding yeast Saccharomyces cerevisiae, Cdc42p promotes different types of polarized growth at several stages of the life cycle (Johnson, 1999; Park and Bi, 2007). During vegetative growth, Cdc42p is essential for establishing polarity and for subsequent bud formation in the late G_1 phase of the cell cycle (Adams *et al.*, 1990). Bud growth is initially targeted to the bud tip (apical growth). As cells enter mitosis, the bud grows over its entire surface (isotropic growth). Haploid yeast cells secrete pheromones to elicit a mating response in cells of the opposite mating type. Cdc42p is involved in pheromone signalling that eventually results in G_1 arrest and the formation of a mating projection (Moskow *et al.*, 2000). Furthermore, Cdc42 is required for the fusion of the haploid cells (Barale *et al.*, 2006). Cell polarization is also required for filamentous growth upon nutrient limitation. Here Cdc42p activates a mitogen-activated protein kinase module in both haploid and diploid cells (Mösch *et al.*, 1996; Mösch *et al.*, 1999). During filamentation, Cdc42p regulates cell morphogenesis and invasion of the substratum (Mösch *et al.*, 2001).

Among the Cdc42p effectors that regulate cell polarization are Ste20p and Cla4p, both members of the p21-activated kinase (PAK) family (Hofmann *et al.*, 2004; Park and Bi, 2007). Cla4p promotes the assembly of the septin ring, which plays a fundamental role in cytokinesis and cell compartmentalization (Weiss *et al.*, 2000; Schmidt *et al.*, 2003; Kadota *et al.*, 2004; Versele and Thorner, 2004). In addition, Cla4p regulates mitotic entry and exit (Höfken and Schiebel, 2002; Seshan *et al.*, 2002). Ste20p activates mitogen-activated protein kinase cascades controlling mating, filamentous growth and the hyperosmotic stress response (Leberer *et al.*, 1992; Liu *et al.*, 1993; Ramer and Davis, 1993; O'Rourke and Herskowitz,

1998; Raitt *et al.*, 2000). Ste20p also contributes to mitotic exit and cell death (Höfken and Schiebel, 2002; Ahn *et al.*, 2005). Furthermore, Cla4p and Ste20p are both involved in vacuolar inheritance (Bartholomew and Hardy, 2009).

Previously, we have demonstrated that Ste20p binds to Erg4p, Cbr1p and Ncp1p, which are all involved in sterol biosynthesis (Tiedje *et al.*, 2007). We also observed genetic interactions between PAKs and ERG4 as well as between PAKs and *NCP1*. Both *ERG4* and *NCP1* are essential in the *cla4Δ* background. Furthermore, *STE20* deletion exacerbates the growth defect of the *ncp1Δ* strain (Tiedje *et al.*, 2007). Cells lacking either *ERG4* or *NCP1* exhibit defects in bud site selection, apical bud growth, cell wall assembly, mating, filamentous growth and mitotic exit (Ni and Snyder, 2001; Keniry *et al.*, 2004; Tiedje *et al.*, 2007). Notably, Ste20p and Cla4p also play important roles in these processes. No phenotypic changes were observed for the *cbr1Δ* strain. By contrast, inactivation of *CBR1* and *NCP1* results in lethality. The large majority of these cells have abnormal bud morphology (Tiedje *et al.*, 2007). Other groups also reported a role for sterols in mating (Bagnat and Simons, 2002; Jin *et al.*, 2008) and it has been suggested that sterol biosynthesis may increase during formation of a mating projection (Kozminski *et al.*, 2006). Furthermore, homologues of oxysterol-binding proteins, a family of proteins that regulate the synthesis and transport of sterols, were found to participate in Cdc42p-dependent polarity (Kozminski *et al.*, 2006). Taken together, these observations suggest that sterol synthesis may play a crucial role in cell polarization and in the function of PAKs and sterol biosynthetic proteins in the same pathway(s). Therefore, it is conceivable that the Cdc42p effectors Ste20p and Cla4p may influence sterol metabolism.

Sterols are important lipid components of eukaryotic membranes that determine different membrane characteristics. Many aspects of sterol homeostasis are conserved between yeasts and humans; and ergosterol, the predominant sterol of yeast, is structurally

and functionally related to sterols of higher eukaryotes (Sturley, 2000). Ergosterol is synthesized primarily in the endoplasmic reticulum through a complex pathway involving numerous steps (Daum *et al.*, 1998). Ergosterol is transported from the endoplasmic reticulum to other organelles, especially to the plasma membrane, where it is greatly enriched (Schneiter *et al.*, 1999). As an excess or lack of free cellular sterol is detrimental, sterol homeostasis is regulated at many stages, including synthesis, uptake, intracellular transport and storage as steryl esters (SE) in cytoplasmic lipid particles. In budding yeast, SE formation is catalyzed by two homologous acyl-CoA:sterol acyltransferases, Are1p and Are2p (Yang *et al.*, 1996; Yu *et al.*, 1996). Both enzymes localize to the endoplasmic reticulum, but differ in their regulation and substrate specificity. Are2p is the major SE synthase under aerobic conditions and esterifies almost exclusively ergosterol (Zweytick *et al.*, 2000; Ptacek *et al.*, 2005). By contrast, Are1p exhibits increased activity under hypoxic conditions and prefers precursor sterols as substrates (Yu *et al.*, 1996; Zweytick *et al.*, 2000).

A large-scale screening revealed Are2p phosphorylation by Ste20p (Ptacek *et al.*, 2005). Therefore, it is conceivable that Ste20p may regulate the activity of this SE-synthesizing enzyme. Considering the importance of sterols for cell polarization, and the interactions between PAKs and proteins catalyzing sterol synthesis and storage, it is tempting to speculate that Ste20p and Cla4p may influence sterol homeostasis. In this work, we show that sterol levels are increased in cells lacking either *STE20* or *CLA4*. The absence of *CLA4* also leads to higher amounts of SE. Furthermore, *CLA4* expression from a multicopy plasmid results in reduced activity of Are2p, the major enzyme of SE formation under aerobic conditions. These data suggest that Ste20p and Cla4p may negatively influence sterol homeostasis.

Materials and Methods

Yeast strains, plasmids and growth conditions

All yeast strains used in this study are in the YPH499 background and are listed in Table 1. Yeast strains were grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) medium or in synthetic complete (SC) medium (Sherman, 1991). For induction of the *GAL1* promoter, yeast cells were grown in 1% yeast extract and 2% peptone or SC media containing 3% raffinose instead of glucose. Galactose (final concentration 2%) was added to induce the *GAL1* promoter. Yeast strains were constructed using PCR-amplified cassettes (Longtine *et al.*, 1998; Knop *et al.*, 1999). All constructs used in this work are listed in Table 2.

Split-ubiquitin technique

For the split-ubiquitin interaction assays, 10^5 wild-type cells carrying the split-ubiquitin plasmids were spotted onto SC-His/Leu plates to select for the plasmids and onto SC-His/Leu/Ura plates to monitor protein–protein interactions, and were grown for 2 days at 30 °C.

Protein analysis

Protein concentration was determined, as described previously (Lowry *et al.*, 1951), using BSA as a standard. Proteins were precipitated using trichloroacetic acid and solubilized in 0.1% SDS and 0.1 m NaOH before quantification.

Glutathione S-transferase (GST) and GST-Ste20 were expressed in *E. coli* BL21 (DE3) and purified using glutathione-sepharose (GE Healthcare, Chalfont St Giles, UK). The immobilized GST proteins were incubated with a yeast lysate of *ARE1*-9myc, *ARE2*-9myc and *CYC8*-9myc, respectively, for 90 min at 4 °C in lysis buffer (20 mm Tris, pH 7.5,

100 mm NaCl, 10 mm EDTA, 1 mm EGTA, 5% glycerol, 1% Nonidet P-40, 1% BSA). After five washes with lysis buffer, the associated proteins were eluted with sample buffer and analyzed by immunoblotting. The mouse anti-Myc (9E10) mAb and the rabbit polyclonal anti-Cdc11p IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse anti-HA (12CA5) was obtained from Roche Diagnostics (Mannheim, Germany) and peroxidase-conjugated secondary IgG was obtained from Pierce (Rockford, IL, USA).

Pheromone response and apical growth assays

For the pheromone response assay, cells grown to the logarithmic phase were incubated with 1 $\mu g \cdot mL^{-1}$ of α-factor in dimethylsulfoxide, or with dimethylsulfoxide alone, for 150 min. Formation of a mating projection in at least 95% of the cells was confirmed microscopically.

For the apical bud growth assay, cells carrying the plasmid pMT485 (GAL1-CLN1-3HA) were grown overnight in selective medium. Exponentially growing cells were induced with galactose for 4 h and fixed with 4% formaldehyde (final concentration) for microscopic examination.

Microscopy

For microscopic examination, cells were fixed with 4% formaldehyde (final concentration) and analyzed using a Zeiss Axiovert 200M fluorescence microscope equipped with a 100× Plan oil-immersion objective. Images were captured using a Zeiss AxioCam MRm CCD camera.

Lipid extraction and analysis

Total cellular lipids were extracted as described previously (Folch *et al.*, 1957). Individual sterols were identified and quantified using GLC/MS after alkaline hydrolysis of lipid extracts (Quail and Kelly, 1996). The protein concentration of 10 mL of culture with an attenuance at 600 nm of 1 was determined and cells were incubated for 2 h at 90 °C together with 0.6 mL of methanol, 0.4 mL of 0.5% pyrogallol dissolved in methanol, 0.4 mL of 60% aqueous KOH and 10 μg of cholesterol dissolved in ethanol as an internal standard. Lipids were extracted three times with n-heptane and the combined extracts were taken to dryness under a stream of nitrogen. Then, lipids were dissolved in 10 μL of pyridine, and after adding 10 μL of N,O-bis(trimethylsilyl)–trifluoroacetamide (Sigma), samples were diluted with ethyl acetate to an appropriate concentration. GLC/MS analysis of silylated sterol adducts was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA, USA), equipped with an HP 5972 mass selective detector and an HP 5-MS column (cross-linked 5% phenyl methyl siloxane; dimensions 30 m × 0.25 mm × 0.25 μm film thickness). Aliquots of 1 μL were injected in the splitless mode at an injection temperature of 270 °C with helium as a carrier gas, at a flow rate of 0.9 $mL \cdot min^{-1}$ in constant flow mode. The following temperature program was used: 1 min at 100 °C, 10 $°C \cdot min^{-1}$ to 250 °C, and 3 $°C \cdot min^{-1}$ to 310 °C. Mass spectra were acquired in scan mode (scan range 200–259 atomic mass units) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

For quantification of SE, lipid extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (Automatic TLC Sampler 4; CAMAG, Muttenz, Switzerland) and chromatograms were developed in an ascending manner using a two-step developing system. First, light petroleum/diethyl ether/acetic acid (25 : 25 : 1, v/v/v) was used as mobile phase and plates were developed to half-distance. Then the plates were dried briefly and further developed to the top of the plate using the second mobile phase consisting of light

petroleum/diethyl ether (49 : 1, v/v). To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g of $MnCl_2 \cdot 4H_2O$, 60 mL of water, 60 mL of methanol and 4 mL of concentrated sulfuric acid, briefly dried and heated at 100 °C for 20 min. SE were then quantified by densitometric scanning at 400 nm using a Shimadzu dual-wavelength chromatoscanner CS930, with cholesteryl ester as the standard.

Acyl-CoA:ergosterol acyltransferase assay

The acyl-CoA:ergosterol acyltransferase assay was performed in a final volume of 100 μL containing 6 nmol of [^{14}C]oleoyl-CoA (88 000 disintegrations per minute), 0.025 mm ergosterol, 0.5 mm CHAPS, 100 mm KH_2PO_4 (pH 7.4), 1 mm dithiothreitol and 200 μg of protein from the homogenate of cells grown to logarithmic phase (Yang *et al.*, 1997). This method relies on the measurement of the amount of radiolabeled steryl esters formed during the assay relative to the substrate employed under standardized conditions. Incubations were carried out for 30 min at 30 °C and terminated by the addition of 300 μL of chloroform/methanol (2 : 1, v/v). Lipids were extracted twice for 10 min with shaking using 300 μL of chloroform/methanol (2 : 1; v/v), each. The organic phases were combined and washed twice using methanol/water/chloroform (47 : 48 : 3, v/v/v). The extraction efficiency of the substrate formed was > 95%. The organic phase was taken to dryness under a stream of nitrogen. Lipids were dissolved in 30 μL of chloroform/methanol (2 : 1, v/v), separated by TLC (as described above) and visualized on TLC plates by staining with iodine vapor. Bands of steryl esters were scraped off, and radioactivity was measured by liquid scintillation counting using an LSC Safety Cocktail (Baker, Deventer, the Netherlands) and 5% water as a scintillation mixture.

Results

Cells lacking either STE20 or CLA4 exhibit increased sterol levels

Sterol biosynthesis plays an important role in cell polarization. Here, we examined whether the Cdc42p effectors Ste20p and Cla4p contribute to the regulation of sterol biosynthesis. To achieve this, lipids were extracted from the wild-type yeast and cells lacking either *CLA4* or *STE20* and sterols were analyzed using GLC/MS. All major sterols were increased in both the *ste20Δ* and the *cla4Δ* mutants (Table 3). In these deletion strains, the amounts of ergosterol and total sterols were approximately 1.3-fold higher compared with those in the wild-type strain ($P < 0.05$). *cla4Δ* cells grow at a rate comparable to that of the wild type but have a grossly abnormal morphology, including highly elongated buds (Cvrckova *et al.*, 1995) (Figure 1A). Cla4p is involved in the degradation of Swe1p, which regulates the switch from apical to isotropic bud growth (Sakchaisri *et al.*, 2004). In the absence of *CLA4*, Swe1p accumulates and cells display elongated buds. In contrast, the *cla4Δswe1Δ* double mutant exhibits normal morphology and cell size (Longtine *et al.*, 2000; Mitchell and Sprague, Jr., 2001) (Figure 1A). Whereas *SWE1* deletion did not affect sterol levels, we observed a higher sterol concentration for the *cla4Δswe1Δ* strain compared with the *swe1Δ* single mutant (Table 3) ($P < 0.05$). Thus, the abnormal morphology of *cla4Δ* cells, and the observed higher amounts of sterol, do not seem to be linked.

It was also tested whether expression of either *STE20* or *CLA4* from a multicopy plasmid has an effect on sterol biosynthesis. Cells carrying *STE20* on a multicopy vector had reduced levels of ergosterol and total sterol (Table 4) ($P < 0.05$). As *STE20* deletion had the opposite effect on the amounts of sterol (Table 3), these data suggest that Ste20p may negatively modulate sterol synthesis. *CLA4* expressed from a multicopy plasmid did not affect the concentration of individual and total sterols (Table 4). As shown in Figure 1B, cells

expressing multicopy *STE20* and *CLA4* displayed normal morphology. Compared with the wild-type cells shown in Table 3, wild-type cells carrying the plasmid pRS425 exhibited higher sterol levels (Table 4). Notably, cells harboring plasmids were grown in selective medium, in contrast to the strains analyzed in Table 3, which were incubated in YPD medium. The different composition of these types of media probably accounts for the difference in sterol levels.

GLC/MS, employed here, not only determines the amount of free unesterified sterols in membranes but also the amount of sterols derived from SE that are hydrolyzed in the course of this preparation (see the Materials and Methods). In contrast, lipid analysis by TLC distinguishes between free and esterified sterols. Notably, we also observed higher levels of free sterols in the *ste20Δ* and the *cla4Δ* strains using TLC (Figure 2). This not only confirms the results obtained by GLC/MS, but also suggests that the amounts of free ergosterol in membranes are increased in cells lacking either *STE20* or *CLA4*.

We previously reported that Ste20p binds to the sterol biosynthetic enzymes Erg4p, Cbr1p and Ncp1p (Tiedje *et al.*, 2007). As the data presented here suggest that not only Ste20p, but also Cla4p, modulates sterol synthesis, we tested whether Cla4p forms a complex with these proteins as well. Using the split-ubiquitin system (Johnsson and Varshavsky, 1994; Wittke *et al.*, 1999), Cla4p, in contrast to Ste20p, did not bind to Erg4p, Cbr1p or Ncp1p (Figure 3A,B). Notably, Cla4p forms a complex with Bem1p in this assay, an interaction that has been reported previously (Gulli *et al.*, 2000; Bose *et al.*, 2001). This demonstrates that the *CLA4* split-ubiquitin construct is suitable for the detection of protein–protein interactions.

Sterols play an important role in cell polarity, in particular during mating (Bagnat and Simons, 2002; Tiedje *et al.*, 2007; Jin *et al.*, 2008), and it has been suggested that the degree of sterol biosynthesis may increase in response to pheromone (Bagnat and Simons, 2002). To test this hypothesis, we analyzed sterols from cells grown in the presence of α-factor and the

solvent dimethylsulfoxide alone. Notably, we did not observe a significant change of the sterol pattern during cell polarization (Table 5). Thus, pheromone signalling does not seem to have an effect on biosynthesis of the major sterols.

Cla4p negatively influences SE formation

We also examined the potential link between the Cdc42 effectors Ste20p and Cla4p and the SE synthases Are1p and Are2p. To start with, it was tested (using a pull-down assay) whether Ste20p forms a complex with Are1p. Indeed, epitope-tagged Are1p expressed in yeast bound specifically to recombinant Ste20p from Escherichia coli (Figure 4). Are2p, the major SE synthase under aerobic conditions, also interacted with Ste20p in this assay (Figure 4). The fact that the unrelated protein Cyc8p did not bind to recombinant Ste20p (Figure 4) indicates that the interaction between Ste20p and the SE synthases Are1p and Are2p is specific. Binding between Cla4p and Are1p or Are2p was not observed in a similar set of experiments (data not shown).

The *are1Δare2Δ* strain, as well as the corresponding single mutants, do not exhibit an obvious phenotype under standard growth conditions (Yu *et al.*, 1996). As Ste20p phosphorylates Are2p (Ptacek *et al.*, 2005) and binds to both SE synthases, we specifically tested whether Are1p and Are2p have a role in cell polarity. Bud site selection, mating and filamentous growth was normal in cells lacking *ARE1* and *ARE2* (data not shown), but apical bud growth following G_1 cyclin overexpression was affected (Figure 5). During budding, the cyclin-dependent kinase Cdc28p promotes polarized apical growth when coupled to the G_1 cyclins and isotropic growth when associated with mitotic cyclins (Lew and Reed, 1993). The apical growth phase can be prolonged by G_1 cyclin overexpression, resulting in hyperelongated buds (Lew and Reed, 1993) (Figure 5A,B). Cells deleted for genes encoding cell-polarity proteins, such as Ste20p, form fewer hyperpolarized buds in response to

overexpression of the G_1 cyclin *CLN1* (Nelson *et al.*, 2003; Tiedje *et al.*, 2007) (Figure 5B). To test whether Are1p and Are2p are involved in apical bud growth, we overexpressed *CLN1* in the corresponding deletion strains and scored for the presence of hyperelongated buds. The deletion of either *ARE1* or *ARE2* resulted in a smaller number of cells with an elongated bud (Figure 5B). A further decrease was observed for the *are1Δare2Δ* double mutant. Immunoblot analysis revealed that the mutant and wild-type cells expressed comparable levels of galactose-induced *CLN1* (Figure 5C). Together, these data suggest that Are1p and Are2p both have a role in apical growth during *CLN1* overexpression.

We next examined whether Ste20p and Cla4p have a role in SE formation. As Are1p activity is negligible under aerobic conditions and difficult to determine, we focused on Are2p. To measure only Are2p-specific effects, the following experiments were performed in cells lacking *ARE1*. First of all, we determined SE levels in the absence of *STE20* and *CLA4*. Whereas *STE20* deletion had no significant effect on SE levels, increased amounts of SE were observed in *cla4Δ* cells (Figure 6A). Expression of either *STE20* or *CLA4* from multicopy plasmids did not alter the levels of SE (Figure 6B). A higher concentration of SE in *cla4Δ* cells could be explained by a negative regulation of Are2p activity by Cla4p. Alternatively, Are2p activity may be normal in cells lacking *CLA4*, and increased amounts of SE might simply be a consequence of higher sterol levels in the cla4Δ mutant. To distinguish between these possibilities, we tested whether Are2p activity depends on *CLA4* and also on *STE20*. To achieve this, the in vitro activity of acyl-CoA:ergosterol acyltransferase was determined in an are1 deletion background. The enzyme activity in the ste20Δ strain and the cla4Δ strain was indistinguishable from that in the wild-type strain (Figure 7A). Therefore, it seems likely that increased sterol biosynthesis in *cla4Δ* cells results in higher amounts of SE without affecting Are2p activity. Interestingly, *CLA4* expression from a multicopy plasmid led to a marked

decrease of Are2p enzyme activity (Figure 7B). Taken together, these data suggest that Cla4p negatively influences sterol biosynthesis and storage.

Discussion

Sterols play an important, but ill-defined, role in cell polarity. It has been suggested that sterol synthesis might increase during polarization and that Cdc42p effectors, such as Cla4p and Ste20p, may control sterol biosynthesis (Tiedje *et al.*, 2007). In this work, we showed that cells lacking either *STE20* or *CLA4* have increased levels of sterols and that expression of *STE20* from a multicopy plasmid lowers sterol concentrations, suggesting that Ste20p and Cla4p may inhibit sterol biosynthesis. Notably, we observed (using TLC and GLC/MS) higher amounts of sterols in the *ste20Δ* and the *cla4Δ* strains. TLC separates free sterols and SE, and both can be quantified. In contrast, GLC/MS allows a detailed analysis of individual sterols, but SE are hydrolyzed and the sterol moiety is included in the total sterol pool. Therefore, our data suggest that the concentration of free sterols in membranes is increased in *ste20Δ* and *cla4Δ* cells.

Previously, we reported that Ste20p interacts with Erg4p, Cbr1p and Ncp1p (Tiedje *et al.*, 2007). Possibly, Ste20p modulates sterol biosynthesis through these enzymes. Erg4p catalyzes the final step of ergosterol synthesis (Lai *et al.*, 1994). Ncp1p and Cbr1p transfer electrons from NADH and NADPH, respectively, to various enzymes of the ergosterol biosynthetic pathway, including Erg1p, Erg3p, Erg5p, Erg11p and the Erg25p/Erg26p/Erg27p complex (Reddy *et al.*, 1977; Aoyama *et al.*, 1981; Yoshida, 1988; Aoyama *et al.*, 1989; Kelly *et al.*, 1995; Lamb *et al.*, 1999). As so many steps of ergosterol biosynthesis depend on

electron transfer from Ncp1p and Cbr1p, these two proteins are ideal targets for the regulation of the whole pathway.

A role for Cla4p in sterol synthesis is consistent with the genetic interactions reported previously. The deletion of either *ERG4* or *NCP1* in the *cla4Δ* background is lethal, indicating that *CLA4* and these genes, encoding proteins involved in sterol synthesis, may function in the same pathway(s) (Tiedje *et al.*, 2007). It is not clear how Cla4p could influence sterol synthesis. In contrast to Ste20p, Cla4p does not bind to Erg4p, Cbr1p and Ncp1.

We also showed here that sterol levels during polarization in response to α-factor treatment remain constant. Ste20p is essential for the arrest at G_1 and the formation of a mating projection following pheromone stimulation (Leberer *et al.*, 1992; Ramer and Davis, 1993), and Cla4p also seems to play a minor role in this pheromone signalling (Chasse *et al.*, 2006; Heinrich *et al.*, 2007), but neither protein seems to affect sterol biosynthesis during the formation of a mating projection. Nevertheless, the phenotypes of mutants defective in ergosterol synthesis clearly demonstrate the importance of sterols for polarization during mating (Bagnat and Simons, 2002; Tiedje *et al.*, 2007; Jin *et al.*, 2008). On the other hand, the observation that sterols enrich at the tip of mating projections, where they could anchor polarity proteins, has been a controversial point of discussion (Valdez-Taubas and Pelham, 2003; Proszynski *et al.*, 2006). Our data suggest that the formation of these sterol-rich domains does not involve a rapid increase in sterol synthesis, but rather clustering of existing sterol molecules and/or a highly focused transport towards the tip of mating projections.

In this work, we also examined the role of Cla4p and Ste20p in SE formation. Increased amounts of SE were observed in the absence of *CLA4*. However, SE synthase activity was not affected in this strain. Therefore, higher SE levels are probably the result of increased sterol synthesis in these cells. Interestingly, however, *CLA4* expression from a multicopy plasmid lowers Are2p enzyme activity. Thus, Cla4p has a negative effect, not only

on sterol biosynthesis but also on SE formation. Reduced Are2p activity in cells containing multicopy *CLA4* does not affect the levels of SE. Possibly, the amount of Are2p in the cell is relatively high in relation to its substrate. A reduction of enzyme activity would then not necessarily have an effect on SE levels. Alternatively, it may simply take more time to form SE. In contrast to *CLA4*, *STE20* deletion and expression from a multicopy plasmid, respectively, had no effect on either SE levels or SE synthase activity. Nevertheless, Ste20p phosphorylates Are2p (Ptacek *et al.*, 2005) and we show here that Ste20p forms a complex with Are1p and Are2p. The functional link behind this finding is not clear.

Interestingly, Ste20p and Cla4p also down-regulate sterol uptake by inhibiting the expression of genes involved in this process (Lin and Höfken, manuscript submitted). Therefore, it seems that Ste20p and Cla4p negatively influence several important sterol homeostatic events. Sterol homeostasis is critical for the cell and is linked to cell polarization. The importance of sterol biosynthesis for polarization during vegetative growth, mating and filamentation has previously been demonstrated (Tiedje *et al.*, 2007). In this study, we showed that the SE synthases Are1p and Are2p are also involved in apical bud growth during G_1 cyclin overexpression. Taken together, we propose that Ste20p and Cla4p contribute to cell polarization in part through the modulation of sterol homeostasis. However, it needs to be established under which conditions Ste20p and Cla4p act on sterol homeostasis. A recent report describes activation of the major triacylglycerol lipase, Tgl4p, by the cyclin-dependent kinase Cdc28p (Kurat *et al.*, 2009). This process links lipolysis with cell-cycle progression, including bud growth. Cla4p might control sterol concentration in a similar way in secretory vesicles and in the plasma membrane during bud formation and growth.

Our data also raise the question of how sterols contribute to cell polarization at the molecular level. Two mechanisms are conceivable. Sterols have a crucial function in endocytosis (Pichler and Riezman, 2004), which in turn is required for the establishment and

maintenance of cell polarity (e.g. by counteracting lateral diffusion of polarized proteins within the membrane) (Valdez-Taubas and Pelham, 2003; Marco *et al.*, 2007). Alternatively, sterols may be important in the association of proteins involved in establishing cell polarity with the plasma membrane, which occurs independently of endocytosis. It has been suggested that sterol-rich domains are compartmentalized in the plasma membrane and serve as an anchor for proteins involved in establishing cell polarity (Proszynski *et al.*, 2006). However, the existence and biochemical nature of such domains is unclear (Bagnat and Simons, 2002; Valdez-Taubas and Pelham, 2003; Proszynski *et al.*, 2006) and further investigations will be required to elucidate the role of sterols in cell polarization in more detail.

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Tables

Table 1. Yeast strains used in this study

Name	Genotype	Source or references
MLY2	YPH499 *are1Δ::klTRP1*	This study
MLY3	YPH499 *are2Δ::klTRP1*	This study
MLY6	YPH499 *are1Δ::klTRP1 are2Δ::HIS3MX6*	This study
MLY20	YPH499 *HIS3MX6-pGAL1-ARE2-9myc-klTRP1*	This study
MLY21	YPH499 *are1Δ::HIS3MX6 ste20Δ::klTRP1*	This study
MLY28	YPH499 *HIS3MX6-pGAL1-ARE1-9myc-klTRP1*	This study
MLY84	YPH499 *are1Δ::HIS3MX6 cla4Δ::kanMX6*	This study
MLY115	YPH499 *ARE1-9myc-HIS3MX6*	This study
THY310	YPH499 *ste20Δ::klTRP1*	Tiedje *et al.*, 2007
THY609	YPH499 *cla4Δ::kanMX6*	This study
THY665	YPH499 *swe1Δ::HIS3MX6*	This study
THY685	YPH499 *swe1Δ::HIS3MX6 cla4Δ::kanMX6*	This study
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter, 1989

Table 2. Plasmids used in this study

Name	Description	Source of reference
pGST-Ste20	pDEST15 carrying *STE20*	Tiedje *et al*., 2007
pML70	pRS313carrying *pMET25-CLA4-CUbiquitin-RURA3*	This study
pMT485	YCp50carrying *pGAL1-CLN1-3HA*	Tiedje *et al*., 2007
pRS425	2µm, *LEU2*-based yeast-*E. coli* shuttle vector	Sikorski and Hieter, 1989
pTH163	pRS425 carrying *CLA4*	This study
pTH197	pRS313 carrying *pMET25-STE20-CUbiquitin-RURA3*	Tiedje *et al*., 2007
pTH338	pADNX carrying *pADH1-NUbiquitin-CBR1*	Tiedje *et al*., 2007
pTH339	pADNX carrying *pADH1-NUbiquitin-ERG4*	Tiedje *et al*., 2007
pTH340	pADNX carrying *pADH1-NUbiquitin-NCP1*	Tiedje *et al*., 2007
pTH344	pADNX carrying *pADH1-NUbiquitin-BEM1*	Tiedje *et al*., 2007
pTH345	pTH345 pADNX carrying *pADH1-NUbiquitin-UBC6*	Tiedje *et al*., 2007
pTH263	pRS425 carrying *STE20*	This study

Table 3. Sterol analysis of cells lacking *STE20* and *CLA4*, respectively. Data are mean values with standard deviation from at least 2 independent experiments.

µg of sterol per mg of protein					
	wild type	*ste20*Δ	*cla4*Δ	*swe1*Δ	*cla4*Δ*swe1*Δ
Ergosterol	15.21 ± 0.66	20.1 ± 1.67	19.58 ± 0.57	15.15 ± 0.98	18.18 ± 1.11
Zymosterol	0.62 ± 0.14	0.84 ± 0.06	0.76 ± 0.11	0.73 ± 0.06	1.14 ± 0.31
Fecosterol	0.48 ± 0.16	0.64 ± 0.04	0.65 ± 0.07	0.45 ± 0.04	0.89 ± 0.30
Lanosterol	0.28 ± 0.09	0.45 ± 0.15	0.38 ± 0.06	0.26 ± 0.08	0.35 ± 0.07
Total sterol	16.59 ± 0.59	22.03 ± 1.65	21.37 ± 0.78	16.49 ± 1.09	20.56 ± 1.52

Table 4. Sterol analysis of cells overexpressing *STE20* and *CLA4*, respectively. Data are mean values with standard deviation from at least 2 independent experiments.

µg sterol/mg protein			
	pRS425	pRS425-*STE20*	pRS425-*CLA4*
Ergosterol	26.67 ± 2.12	21.32 ± 0.86	27.44 ± 0.90
Zymosterol	2.36 ± 0.11	1.99 ± 0.16	2.53 ± 0.22
Fecosterol	0.92 ± 0.05	0.91 ± 0.09	1.20 ± 0.08
Lanosterol	0.60 ± 0.27	0.31 ± 0.10	0.68 ± 0.10
Total sterol	30.55 ± 2.10	24.53 ± 1.01	31.85 ± 1.11

Table 5. Sterol composition of cells in response to Γ -factor. Mean values with standard deviation from 5 independent experiments.

µg sterol/mg protein		
	Dimethylsulfoxide	α-factor
Ergosterol	21.89 ± 2.00	20.84 ± 1.61
Zymosterol	1.20 ± 0.14	1.24 ± 0.20
Fecosterol	0.48 ± 0.15	0.49 ± 0.15
Lanosterol	0.61 ± 0.32	0.55 ± 0.29
Total sterol	24.18 ± 1.94	23.12 ± 1.71

Figures

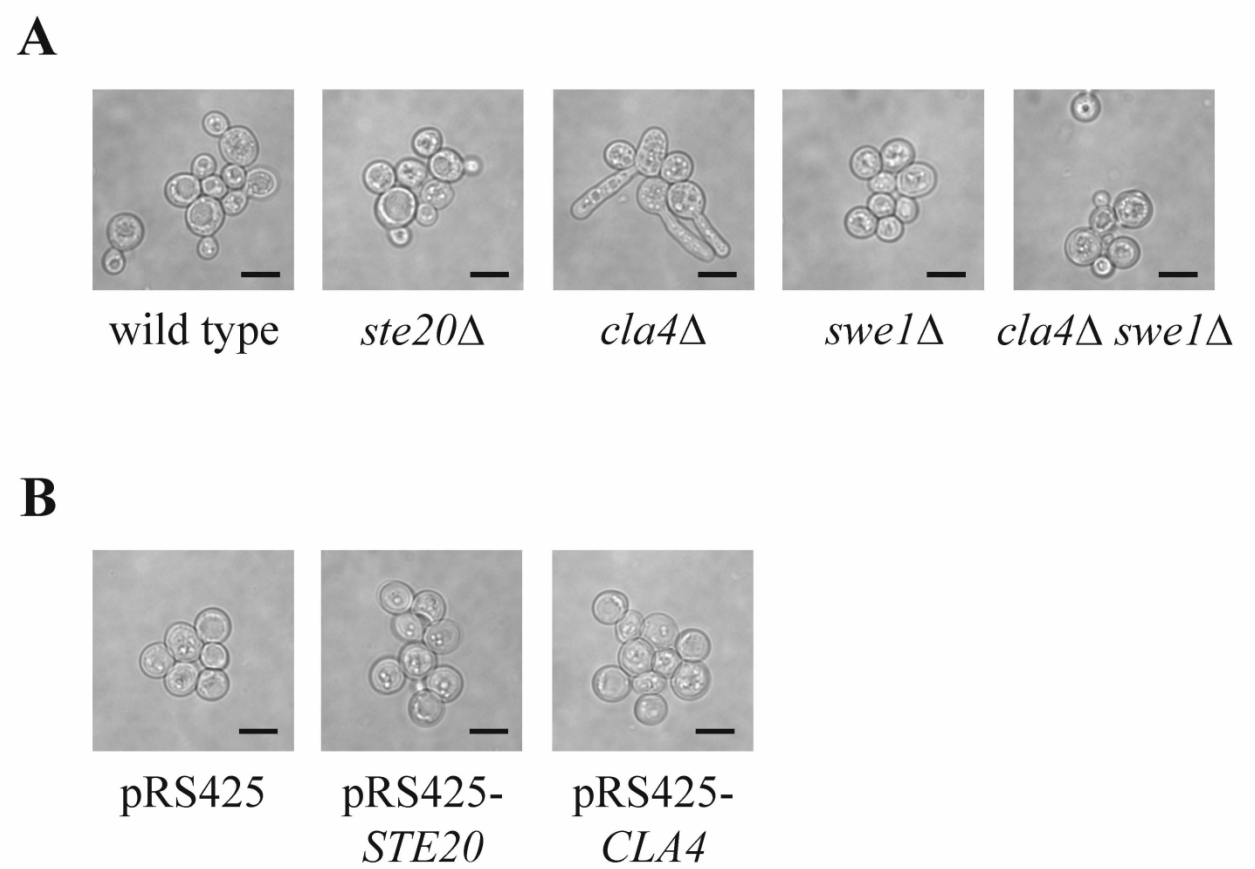

Figure 1

Cell morphology of the strains used in this study.

(A) Morphology of deletion strains. The indicated strains were grown in YPD to stationaryphase. Cells were then fixed with formaldehyde for microscopical examination. Bars: 5 µm (B) Expression of either *STE20* or *CLA4* from a multi-copy plasmid does not affect morphology. Cells were grown in minimal medium to stationary phase. Bars: 5 µm.

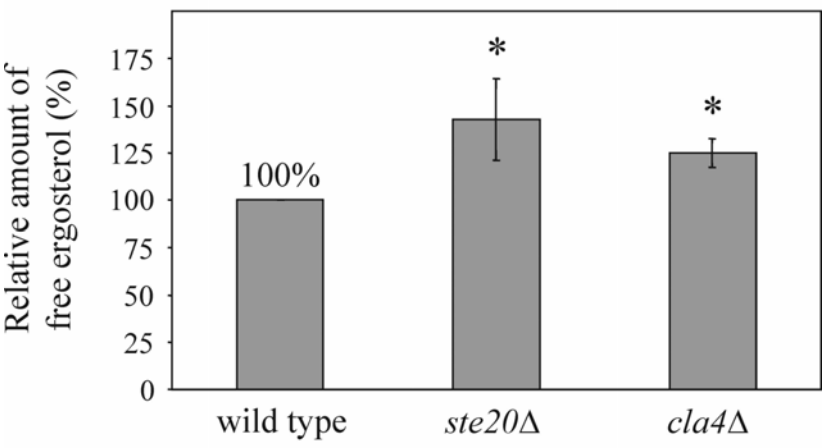

Figure 2

Cells lacking either *STE20* or *CLA4* have increased levels of free sterol

The indicated strains were grown to stationary phase, lipids were extracted and separated by TLC. Data are from 2 independent experiments. *, $P < 0.05$ compared to the wild type

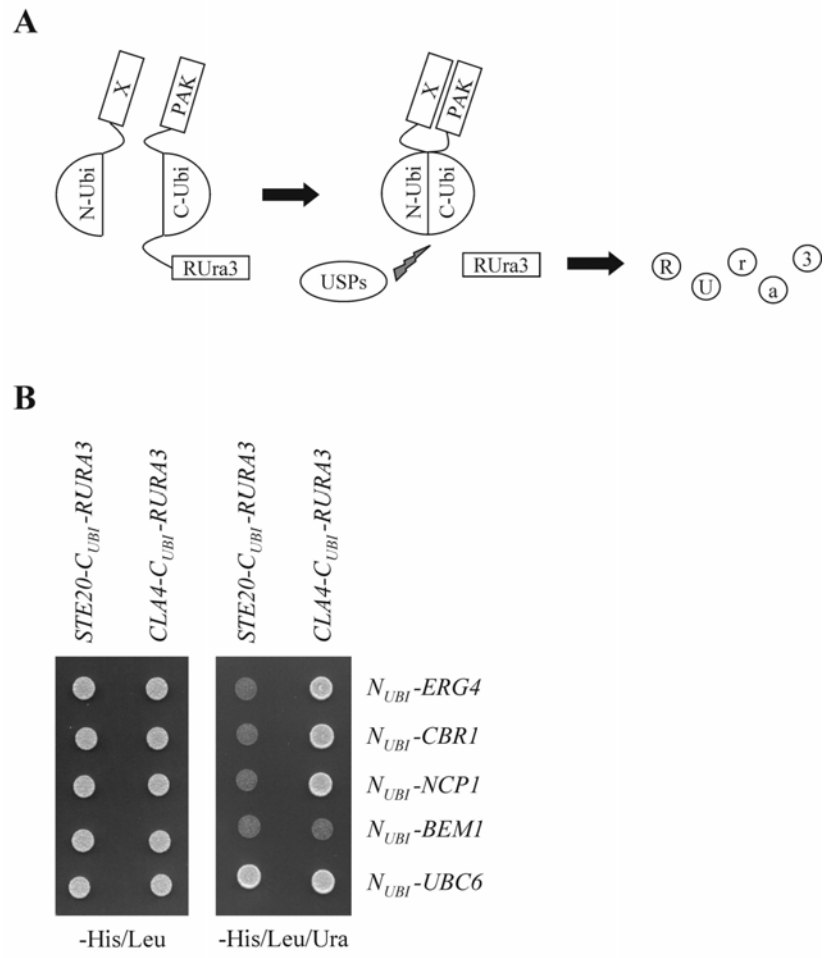

Figure 3

Cla4p does not bind to Erg4p, Cbr1p or Ncp1p.

(A) The split-ubiquitin system. The N-terminal and C-terminal halves of ubiquitin (N-Ubi and C-Ubi) alone do not assemble. If a protein "X", fused to N-Ubi, binds to the PAKs Ste20p or Cla4p, linked to C-Ubi, both halves of ubiquitin are brought into close proximity. This reconstituted quasi-native ubiquitin is recognized by ubiquitin-specific proteases (USPs), which cleave off the reporter RUra3, which is fused to the PAK. Released RUra3, a modified version of the enzyme Ura3 with an arginine at the extreme N-terminus, is targeted for degradation by the enzymes of the N-end rule. A protein-protein interaction, therefore, results in non-growth on medium lacking uracil. (B) In contrast to Ste20p, Cla4p does not bind to Erg4p, Cbr1p and Ncp1p. 10^5 cells of the indicated plasmid combinations were spotted either onto medium lacking histidine and leucine to select for the plasmids or onto medium lacking histidine, leucine and uracil to monitor protein interactions. The unrelated genes *STE14* and *UBC6* served as negative controls.

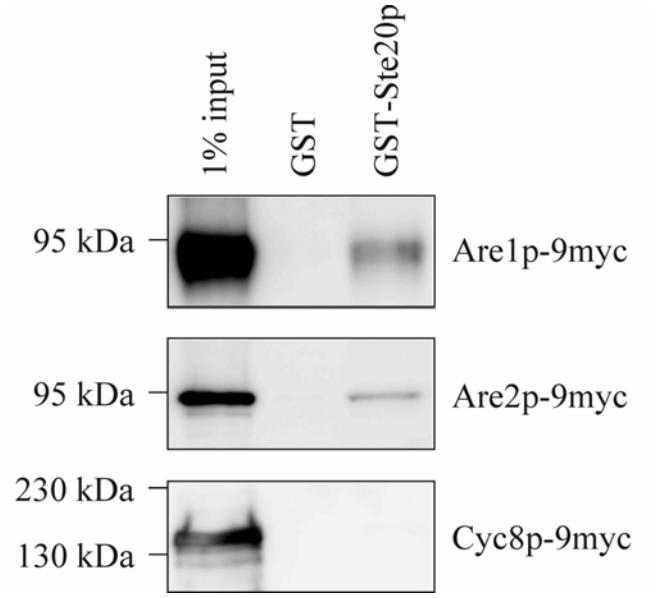

Figure 4

Ste20p interacts with both SE synthases.

Purified GST and GST-Ste20p were immobilized on glutathione-sepharose beads and incubated with a yeast lysate of *ARE1-9myc*, *ARE2-9myc* and *CYC8-9myc* cells, respectively. Eluted proteins were analyzed by immunoblotting using anti-myc antibodies. One percent of the input is shown. Predicted molecular weights: Are1p-9myc: 81 kDa; Are2p-9myc: 83 kDa; Cyc8p-9myc: 116 kDa.

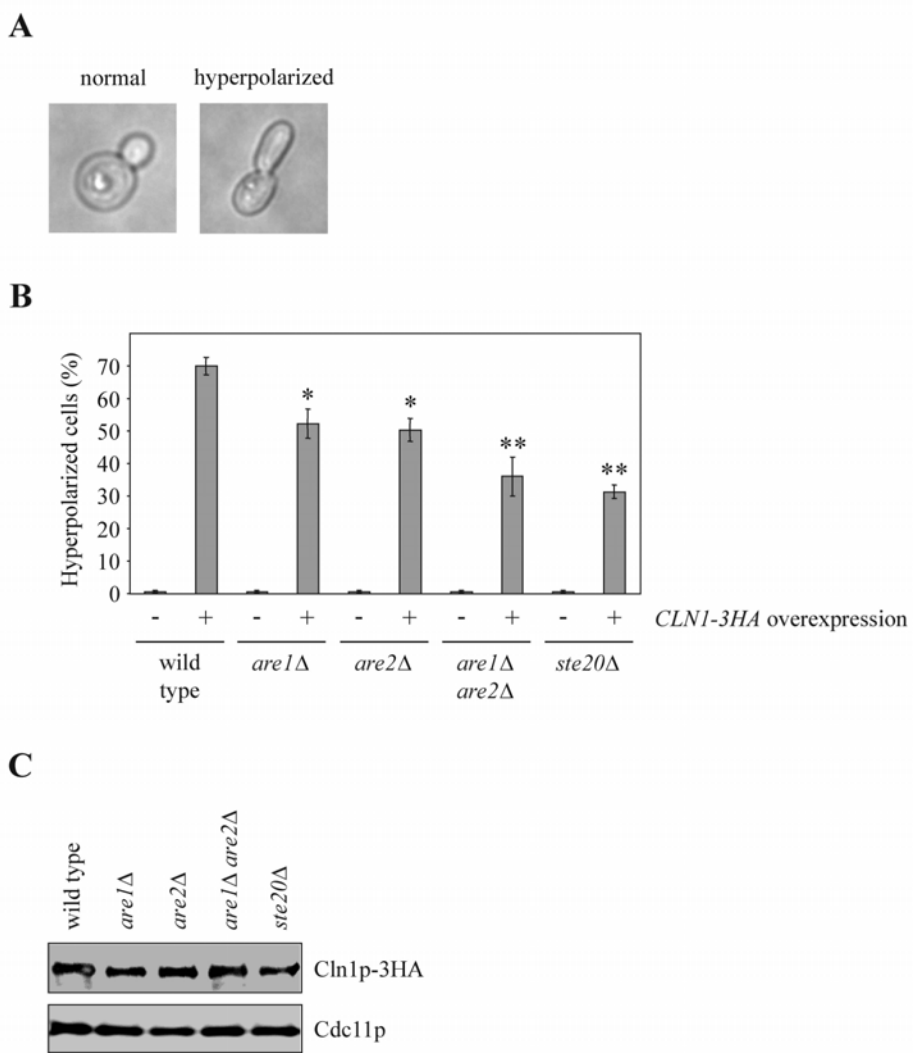

Figure 5

Are1p and Are2p have a role in apical bud growth.

(A) Morphology of normal and hyperelongated cells. Exponentially growing cells carrying *pGAL1-CLN1-3HA* on a plasmid were induced by the addition of galactose for 4 hours. Cells were then fixed with formaldehyde. (B) Are1p and Are2p are involved in apical growth. Cells carrying either carrying *pGAL1-CLN1-3HA* on a plasmid or the empty vector were treated as in (A). The percentage of cells with a hyperpolarized bud was determined in 3 independent experiments (n >100 each). *, $P<0.05$ compared to the wild type with *CLN1* overexpression; **, $P < 0.01$ compared to the wild type with *CLN1* overexpresion and $P < 0.05$ compared to the *are1*Δ and *are2*Δ mutants with *CLN1* overexpresion (C) Wild type and deletion strains express comparable amounts of *CLN1*. Cells from (B) were analyzed by immunoblotting with anti-HA antibodies. Cdc11p was used as loading control.

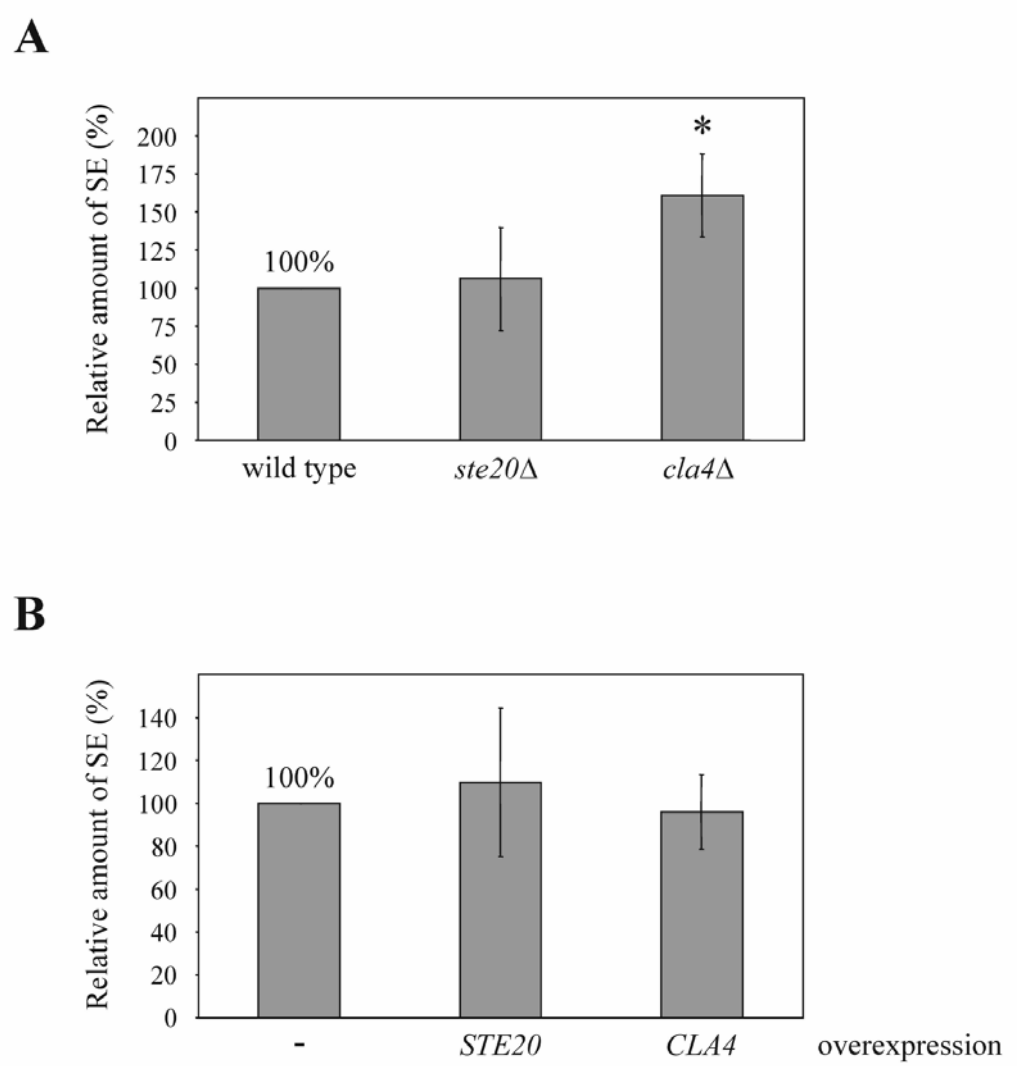

Figure 6

Deletion of *CLA4* results in higher amounts of SE.

(A) SE quantification of cell polarity mutants. Cells of the indicated strains were grown to stationary phase, lipids were extracted and separated by TLC. The amount of SE of the wild type was set at 100%. Data are mean values of 3 independent experiments. *ARE1* was deleted in all strains. *, $P < 0.05$ compared to the wild type (B) Expression of either *STE20* or *CLA4* from a multi-copy plasmid does not affect SE levels. *are1*Δ cells carrying either *STE20* or *CLA4* on a multi-copy plasmid or the vector alone were treated as in (A). Data are from 3 independent experiments.

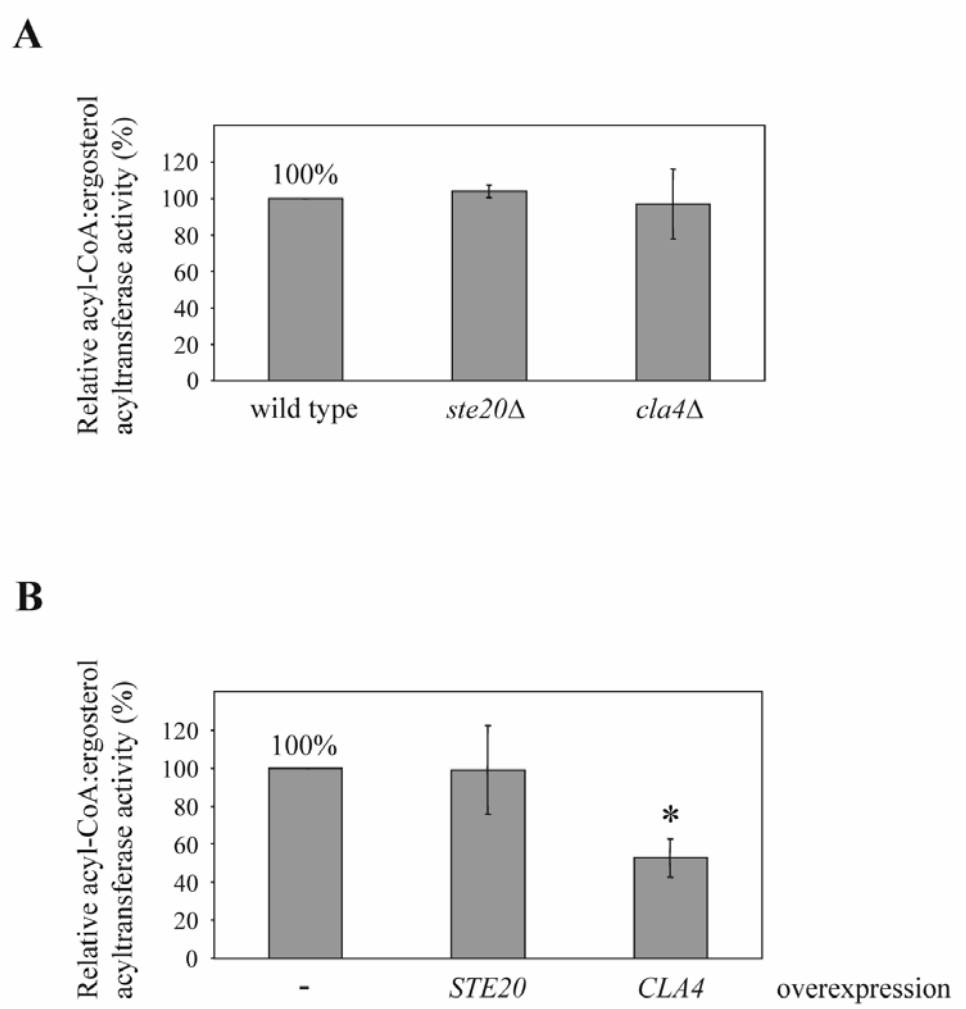

Figure 7

***In vitro* activity of Are2p.**

(A) Acyl-CoA:ergosterol acyltransferase was measured *in vitro* using cell homogenates from the indicated strains. The specific enzyme activity in the wild type was set at 100%. Data are from 2 independent experiments. *ARE1* was deleted in all strains.(B) *CLA4* expression from a multi-copy plasmid results in reduced Are2p activity. *are1*Δ cells carrying either *STE20* or *CLA4* on a multi copy plasmid or the vector alone were treated as in (A). Data are from 2 independent experiments. *, $P < 0.05$ compared to the wild type.

Chapter 4

Analysis of Lipid Particles from Yeast

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Key words: Lipid particles/droplets, phospholipid, triacylglycerol, steryl ester, yeast

Abbreviations:

CWW, cell wet weight; ER, endoplasmic reticulum; GLC, gas liquid chromatography; LP, lipid particle(s)/droplet(s); MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; STE, steryl ester; TAG, triacylglycerol; TLC, thin layer chromatography; RT, room temperature.

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Abstract

Quantitative analysis of components from different subcellular fractions is a key to the understanding of metabolic function as well as to the origin, the biogenesis and the crosstalk of organelles. The yeast is an excellent model organism to address such questions from the biochemical, molecular biological and cell biological viewpoints. A yeast organelle which gained much interest during the last decade is the lipid particle/droplet (LP), a storage compartment for non-polar lipids but at the same time an organelle actively contributing to cellular metabolism. In this chapter, we describe methods and techniques that are commonly used to analyze lipids from LP at the molecular level by thin-layer chromatography, gas liquid chromatography and mass spectrometry. We provide an easy to follow guideline for the isolation of these organelles, the qualitative and quantitative analysis of lipid components and show results obtained with these methods.

1. Introduction

One of the most important prerequisites to establish a well organized and preserved cellular structure is the formation of biological membranes that protect or separate organelle components from the cellular environment, and shield cells from the exterior. Major constituents of membranes are lipid molecules, especially phospholipids, sterols, sphingolipids and glycolipids. Moreover, all types of cells contain storage lipids which are also referred as neutral lipids or non-polar lipids. In most cases, these neutral lipids are stored in a very specific compartment named lipid particle (LP), lipid droplet, lipid body or oil body (Athenstaedt and Daum, 2005). Contrary to other cellular organelles, the LP does not contain a phospholipid bilayer on its surface but rather a phospholipid monolayer with the polar head groups facing the hydrophilic environment and the hydrophobic part associating with the non-polar lipids of the LP core (Athenstaedt *et al.*, 1999;Zweytick *et al.*, 2000). The current hypothesis of LP biogenesis is based on a budding model suggesting that LP derive from the endoplasmic reticulum (ER) (Scow *et al.*, 1980;Blanchette-Mackie *et al.*, 1995;Robenek *et al.*, 2006;Czabany *et al.*, 2007). It has been proposed that neutral lipids formed in the ER accumulate in certain domains which grow and generate the LP core. Once the size of the non-polar lipid droplet increases and exceeds accommodation in the ER phospholipid bilayer, the LP buds off forming an independent organelle.

In the yeast *Saccharomyces cerevisiae*, steryl esters (STE) and triacylglycerols (TAG) are the major neutral lipid components of LP. Under normal growth conditions of the yeast the existence of LP is important although not essential (Sandager *et al.*, 2002). LP are assumed to store excess amounts of fatty acids and sterols in the biological inert form of STE and TAG and thereby avoid possible lipotoxic effects of these components. At the same time,

storage and mobilization of neutral lipids helps the cell to conserve energy and building blocks for membrane biogenesis for conditions of nutritional depletion or cellular stress.

Yeast LP are equipped with a very specific subset of proteins. Many of them being involved in neutral lipid metabolism, e.g., Dga1p, Tgl3p, Tgl4p, Tgl5p, Tgl1p and Yeh2p, or in ergosterol biosynthesis (Erg1p, Erg6p, Erg7p) (Athenstaedt *et al.*, 1999;Rajakumari *et al.*, 2008). Interestingly, some of these proteins such as Erg1p or Dga1p are dually located within the cell, namely in LP and the ER. How such proteins can be localized to two different types of membrane, a monolayer in the LP and a bilayer in the ER, is not yet understood. This observation also raises the question as to the specificity of the membrane lipid composition in these two compartments which might be important to understand the relationship between the two organelles.

In this chapter, we will describe methods commonly used for the analysis of lipids from yeast LP, which can also be adapted to other cell types. These techniques are versatile and can be easily performed. As a starting point for LP analysis, the isolation procedure of this organelle and the quality control of purified organelle samples will be explained. Then, we will describe lipid extraction and quantification of individual lipid components based on thin layer chromatography (TLC), gas liquid chromatography (GLC), mass spectrometry (MS) and GLC/MS.

2. Material

2.1 Equipment and Supplies

1. Microsyringe (Hamilton, Bonaduz, Switzerland) or sample applicator (CAMAG, Automatic TLC Sampler IV, Muttenz, Switzerland)
2. 12 ml Pyrex glass vials with Teflon liner caps
3. Glass tubes with ground neck
4. Dounce Homogenizer and pestle
5. Silica gel 60 TLC plates (Merck, Darmstadt, Germany)
6. Ultra-Clear Centrifuge Tube (Beckman)
7. TLC chamber (Springfield Mill, UK) with saturation paper (e.g., Whatman filter paper)
8. Iodine vapor chamber
9. Incubator (Heraeus)
10. TLC Scanner (Shimadzu chromatoscanner CS-930)
11. Table-top centrifuge (Hettich Rotina 46 R, Heraeus Fresco17)
12. Sorvall RC5 Plus or RC6 Plus, and SLC3000 or SS34 rotors
13. Ultracentrifuge (Sorvall – combi plus) with AH629 swing out rotor
14. GLC-MS (Hewlett-Packard 5890 Gas-Chromatograph)
15. MS LTQ-FT coupled to and Accela UPLC

2.2 Reagents

1. Medium for yeast cultivation:

   YPD (2 % glucose, 2 % peptone and 1 % yeast extract)

   SD (2 % glucose, 0.67 % yeast nitrogen base and amino acid mixture)

   YPO (0.1 % yeast extract, 0.5 % peptone, 0.5 % KH_2PO_4, 0.1 % glucose, 0.2 % Tween 80, 0.1 % oleic acid)
2. Zymolyase-20 T (Seikagaku corporation, Japan)
3. Ficoll 400 (Sigma)
4. Chemiluminescence solution: SuperSignal^{TM} (Pierce Chemical Company, Rockford, IL, USA)
5. Solvents: chloroform and methanol, analytical grade
6. Washing solutions: 0.034 % $MgCl_2$; 2N KCl/MeOH (4:1; v/v); artificial upper phase (chloroform/methanol/water; 3:48:47; per vol.)
7. Charring solution: 0.63 g $MnCl_2 \cdot 4\ H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. H_2SO_4

3. Methods

3.1 Isolation of lipid particles from the yeast

1. LPs are isolated from 4-5 l of full or selective media (2.2.1; see also section 5.5.). Cells are inoculated from a 48 h pre-culture to an OD_{600} of 0.1 and grown to stationary phase at 30 °C with shaking.

2. After harvesting at 5,000 rpm for 5 min in SLC3000, cells are washed with distilled water.
3. Cells (0.5 g CWW/ml) are incubated with dithiothreitol (DTT; 0.66 mg/ml) in buffer SP-A (0.1 M Tris/SO_4, pH 9.4) for 10 min at 30 °C with shaking.
4. Then, cells (0.15 g CWW/ml) are washed and suspended in pre-warmed SP-B (1.2 M sorbitol, 20 mM KH_2PO_4, pH 7.4). Cell walls are enzymatically digested with Zymolyase-20T (Seikagaku Corporation, Japan) at a concentration of 2 mg enzyme per g CWW for at least 1 h at 30°C with shaking.
5. Resulting spheroplasts are washed twice in buffer SP-B and then resuspended in buffer LP-A (12 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2\ H_20$) followed by mechanical disruption in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; 1 mM final) with 30 strokes using a 30 ml Dounce Homogenizer with a loose fitting pestle.
6. The homogenate is centrifuged to remove cell debris in a Sorvall SS34 rotor at 7,000 rpm for 5 min.
7. Supernatants are collected, and steps 6 and 7 are repeated.
8. Combined supernatants are carefully overlaid with buffer LP-A in an Ultra-Clear Centrifuge Tube (Beckman). Ultracentrifugation at 28,000 rpm for 45 min using a swing out rotor AH-629 yields a white layer on top (crude LP) that can be transferred with a moistened spatula to a 15 ml Dounce Homogenizer.
9. After homogenizing with 8 strokes using a loose fitting pestle in the presence of 1 mM PMSF the sample is loaded onto a new ultracentrifuge tube and carefully overlaid with buffer LP-B (8 % Ficoll, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA{\cdot}2\ H_20$). Ultracentrifugation at 28,000 rpm for 30 min results in a top layer containing LP (see notes 5.6).

10. Prior to the last ultracentrifugation step, buffer LP-D (0.25 M sorbitol, 10 mM MES/Tris pH 6.9, 0.2 mM $Na_2EDTA \cdot 2\ H_20$) is filled into a fresh ultracentrifuge tube. The homogenized sample is loaded to the bottom of the tube by injection under the buffer using a syringe.
11. Ultracentrifugation at 28,000 rpm for 30 min leads to a top layer consisting of highly purified LP. After homogenizing the isolated LP in a 5 ml Dounce Homogenizer the samples can be stored at -80 °C until required. If desired the pellet from the last centrifugation step which contains vacuoles can be collected as well.

3.2 Lipid particle analysis

3.2.1 Protein analysis

1. When applying ‘delipidation’ to isolated LP fractions, samples are incubated with 2 volumes of diethyl ether with repeated vigorous shaking. After a high speed centrifugation step using a table top centrifuge the extracted non-polar lipids are withdrawn and remaining traces of diethyl ether are removed under a stream of nitrogen.
2. Proteins are precipitated with trichloroacetic acid, and the resulting pellets are either dissolved in one-fold Laemmli buffer for gel electrophoresis (Laemmli, 1970) or solubilized in 0.1 % SDS/0.1 M NaOH for protein quantification.
3. Proteins are quantified using the method of Lowry et al. (Lowry *et al.*, 1951) with bovine serum albumin as a standard. Typical samples of LP fractions contain approximately 0.2-0.02 µg protein/µl depending on strain and growth conditions.

4. Samples must not be dissolved at temperatures higher than 37 °C as such a treatment would result in hydrolysis of proteins or in the formation of aggregates.
5. SDS-polyacrylamide gel electrophoresis is performed as described by Laemmli (Laemmli, 1970).
6. Commassie Blue staining of gels is usually sufficient to visualize protein bands, though more sensitive staining procedures (Silver staining, inverse protein staining) may be required to detect proteins of low abundance.
7. Western blot analysis is performed according to the method of Haid and Suissa (Haid and Suissa, 1983).
8. A set of antibodies representing typical marker proteins of various cellular organelles is used to check the quality of the isolated LP (Table 1 and related results in Figure 1, below)
9. Peroxidase conjugated secondary antibody (Sigma) and enhanced chemiluminescent signal detection reagents (SuperSignal^{TM}, Pierce Chemical Company, Rockford, IL, USA) are used to visualize immunoreactive bands.
10. When LP samples appear to be contaminated with other subcellular compartments a further purification step can be introduced after the standard LP isolation procedure (see Note 5.2.).

Table 1 (here)

3.2.2 Lipid extraction

1. Lipids from LP are extracted using the method of Folch et al. (Folch J. *et al.*, 1957).
2. In brief, an aliquot of the LP sample (~ 0.1 mg protein) is added to 3 ml $CHCl_3$:MeOH (2:1) in a Pyrex glass tube.
3. Lipids are extracted to the polar organic phase by vortexing at room temperature (RT) for 1 h.
4. It is very important to remove all protein aggregates by discarding the upper aqueous phase as well as the protein interface layer. Alternatively, the lower polar phase can be transferred to a fresh Pyrex tube by using a glass pipette.
5. Proteins and non-polar substances are removed by consecutive washing steps with 0.2 volumes 0.034 % $MgCl_2$, 1 ml 2 N KCl/MeOH (4:1; v/v) and 1 ml of an artificial upper phase ($CHCl_3$:MeOH:H_2O; 3:48:47; per vol.). These solutions are added to the extracts and incubated with shaking for 3 min.
6. After centrifugation for 3 min at 3,000 rpm in a table-top centrifuge the aqueous phase is removed by aspiration.
7. Washing steps are repeated until no protein layer is formed any more.
8. Finally, lipids are dried under a stream of nitrogen and stored at -20 °C.
9. For lipid analyses, 1D and 2D thin layer chromatography (TLC) can be performed to separate different lipid classes and species according to their properties (see 3.2.3 and 3.2.4).

3.2.3 Thin layer chromatographic analysis of phospholipids

1. Phospholipids are separated by TLC due to different properties of their headgroups. TLC plates can be loaded with a Hamilton syringe or with a sample applicator. For the analysis of PI, PC and PE, lipids are separated by one-dimensional (1D) TLC using chloroform/methanol/25 % ammonia solution (50:25:6; per vol.) as a solvent. Separations usually take 50 min/10 cm of distance on TLC plates.
2. A better separation of phospholipids can be achieved by 2D TLC. The sample is applied as single spot to a TLC plate approximately 1-1.5 cm distant from the corner. For the first dimension, chloroform/methanol/25 % ammonia (65:35:5; per vol.) is used as a solvent, and for the second dimension chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.).
3. Phospholipids are visualized by staining with iodine vapor in a saturated chamber for some minutes. For destaining TLC plates are incubated in a heating chamber for a few minutes.
4. Phospholipids can be quantified from TLC plates after removal of the iodine staining. The plate is moistened with deionized water, phospholipid spots are scrapped off and transferred to a phosphate free glass tube with ground neck.
5. The lipid phosphorus of the respective spot can be measured by subjecting the sample to hydrolysis. Therefore, 0.2 ml of conc. H_2SO_4/72 % $HClO_4$ (9:1; v/v) is added to each sample. Hydrolysis is performed at 180°C in a heating block for 30 min. Please note that this step has to be performed in a hood due to formation of acidic fumes!
6. Samples are cooled to RT, and 4.8 ml of freshly prepared 0.26 % ammonium molybdate/ANSA (500:22; v/v) is added. ANSA consists of 40.0 g $K_2S_2O_5$, 0.63 g 8-

anilio-1-naphthalenesulfonic acid and 1.25 g Na_2SO_3 in 250 ml water. Tubes are sealed with phosphate free glass caps and after vigorous vortexing samples are heated to 100 °C for 30 min in a heating chamber.

7. Finally, samples are cooled to RT and shortly centrifuged in a table top centrifuge at 1,000 x g to sediment the silica gel. The intensity of the blue color is a measure for the lipid phosphorus. Samples are measured spectrophotometrically at a wavelength of 830 nm using a blank spot from the TLC plate without phospholipid as a control. Data are calculated from a standard curve using inorganic phosphate at known amounts.

3.2.4 Thin layer chromatographic analysis of neutral lipids

1. Neutral (non-polar) lipids are extracted as described above (3.2.2). The separation of different classes of neutral lipids can be performed by TLC. For the direct densitometric quantification of lipids on the TLC plate authentic standards are used containing defined amounts of the respective lipids.
2. Dried lipid extracts are dissolved in an appropriate volume of $CHCl_3$/MeOH (2:1; v/v) and spotted onto a TLC plate with a Hamilton syringe or a TLC sample applicator (see above).
3. For a most efficient TLC separation of neutral lipids a two step separation system is recommended. Initially, lipids are separated on a 10 cm TLC plate in an ascending manner using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as a solvent until the front has reached two thirds of the plate's height. Then, separation of lipids is continued in the same direction using light petroleum/diethyl ether (49:1; v/v) until the solvent front reaches the top of the plate.

4. After the TLC plate has been dried, sterols can be quantified densitometrically using a TLC scanner (Shimadzu chromatoscanner CS-930) at 275 nm. Longer exposure of the TLC plate to light and oxygen causes oxidation of sterols which should be avoided.
5. Other neutral lipids are irreversibly stained by charring prior to scanning. For this purpose, the TLC plate is incubated approximately 15 sec in a solution of 0.63 g $MnCl_2 \cdot 4H_2O$, 60 ml water, 60 ml methanol, 4 ml conc. sulfuric acid and stained in a heating chamber at 100°C for 30 min. The staining intensity depends on the incubation time. The scanning procedure should be performed directly after charring as the intensity of the spots is fading with time (also see Note 6).
6. Spot of neutral lipids visualized as described above are scanned at a wavelength of 400 nm, and spot areas are quantified relative to standards.

3.2.5 Gas liquid chromatographic analysis of fatty acids

1. Fatty acids are analyzed by gas liquid chromatography after hydrolysis and conversion to methyl esters.
2. For this purpose, lipid extracts (see 3.2.2) are treated with BF_3-methanol as described in the literature (Morrison W.R. and Smith L.M., 1964).
3. In detail, aliquots of lipid extracts (0.1-1 ml) are transferred to glass tubes and dried under a stream of nitrogen. After addition of 1 ml BF_3-methanol, samples are heated to 95°C in a sand bath for 10 min and then cooled to RT. 0.86 ml benzene are added and tubes are heated again in the sand bath at 95 °C for 30 min.
4. After cooling to RT, 1 ml of H_2Odd and 3 ml of light petroleum are added, and samples are vortexed for 30 min. Then, samples are centrifuged at 2,500 rpm for 2 min in a table top centrifuge (Hettich Universal 16), and the upper phase is transferred to a

new Pyrex tube. The lower phase is extracted again as described above for 30 min with vortexing using 3 ml of light petroleum. After centrifugation the organic phases are combined and dried under a stream of nitrogen.

5. Then, samples are dissolved in 100 µl light petroleum and transferred to glass tubes. Finally, fatty acid methyl esters are separated by GLC using following parameters:

GLC	HP 6890
Injector mode	split
Injection volume	1 µl
Column	HP-INNOWax Polyethylene Glycol; 15 m x 0.25 mm i.d. x 0.5 µm film thickness
Carrier	Helium, 5.0
Flow	1.4 ml linear velocity 30 cm/s constant flow
Oven	160 °C (5 min) with 7.5 °C/min to 250 °C (15 min)
Detector	FID mode: constant makeup flow (40.0 ml/ min) Air flow: 400.0 ml / min Hydrogen flow: 35.0 ml / min Makeup gas type: Helium

6. Fatty acids are identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

3.2.6 Gas liquid chromatography/mass spectrometry of sterols

1. Sterol analysis is performed as described previously (Quail and Kelly, 1996).
2. A mixture of 0.6 ml methanol (Merck) and 0.4 ml 0.5 % (w/v) pyrogallol (Fluka; 4 °C freshly dissolved in methanol and 0.4 ml 60 % (w/v) aqueous KOH solution) in a Pyrex tube is prepared. Then, 5 µl of a cholesterol solution (2 mg/ml in ethanol) is added as an internal standard.

3. Samples containing approximately 0.3-0.5 mg protein are added to the mixture and tubes are heated in a water bath at 90 °C for 2 h.
4. Lipids are extracted with 1 ml n-heptane. The upper phase is transferred into a fresh tube, and the lower phase is re-extracted twice. Combined upper phases are dried under a stream of nitrogen, and lipids are dissolved in 10 µl pyridine.
5. Samples are treated immediately prior to analysis with 10 µl N'O'-bis (trimethylsilyl)-trifluoracetamide (SIGMA), incubated at RT for 10 min and diluted with 30 µl ethyl acetate.
6. GLC-MS analysis is carried out using the following parameters:

GLC	HP 5890 SeriesII Plus with Electonic Pressure Control and 6890 automated liquid sampler (ALS)
Injector	Split/splitless 270 °C, mode: splitless. Purge on: 2 min
Injector volume	1 µl
Column	HP 5-MS (Crosslinked 5 % Phenyl Methyl Siloxane); 30 m x 0.25 mm i.d. x 0.25 µm film thickness
Carrier	Helium, 5.0
Flow	0.9 ml linear velocity 35.4 cm/s, constant flow
Oven	100 °C (1 min) with 10 °C/min to 250 °C (0 min) and with 3 °C/min to 310 °C (0 min)
Detector	Selective Detector HP 5972 MSD
Ionization	EI, 70 eV
Mode	Scan, scan range: 200-550 amu, 3.27 scans/s
EM Voltage	Tune Voltage
Tune	Auto Tune

7. Sterols are identified according to their retention time and mass fragmentation pattern using MSD ChemStation, D.03.00.552.

3.2.7 Mass spectrometry of neutral lipids and phospholipids

1. Lipids extracts are prepared as described above and diluted 1:100 in acetonitrile/2-propanol (5:2; v/v), 1 % ammonium acetate, 0.1 % formic acid.
2. 5 µM TAG (species 51:0) and PC (species 24:0) are added as internal standards.
3. For chromatographic separation a thermo hypersil GOLD C18, 100 x 1mm, 1.9 µm column is used with solvent A (water with 1 % ammonium acetate, 0.1 % formic acid) and solvent B (acetonitrile/2-propanol, 5:2, v/v; 1 % ammonium acetate; 0.1 % formic acid).
4. Gradients are established from 35 % to 70 % B for 4 min and then to 100 % B in another step of 16 min. These conditions are held for 10 min with a flow rate of 250 µl/min.
5. Mass spectrometry is performed with the following parameters:

MS	LTQ-FT coupled to and Accela UPLC
Data acquisition	FT-MS full scan at a resolution of 100 k and < 2 ppm mass accuracy with external calibration
Spray voltage	5000 V
Capillary voltage	35 V
Tube lens	120 V
Capillary temperature	250 °C

6. Peak areas are calculated by QuanBrowser for all lipid species identified previously by exact mass (< 2 ppm) and retention time. Calculated peak areas for each species are expressed as % of the sum of all peak areas in the respective lipid class.

4. Results

1. Fig. 1A shows a typical SDS-PAGE analysis of a cell fractionation experiment using the yeast wild type strain BY4741. LP proteins occur at low abundance in the cell, but comprise a distinct set of polypeptides (Leber *et al.*, 1994).

FIGURE 1 here

2. The purity of LP preparation can be tested by Western blot analysis (Fig. 1B) using marker antibodies as described in Table 1. The enrichment of LP can be verified best with anti-Erg1p antibody (Fig. 1B, line 1). Contamination of LP with other organelles such as mitochondria (Por1p), ER (Sec61p) or vacuole (Prc1p) can be largely excluded since signals with the respective antibodies in the LP fraction are negligible.
3. Phospholipid analysis of LP has been described previously in the literature (Leber *et al.*, 1994). In brief, total phospholipids from LP contained approximately 36 % PC, 20 % PE, 32 % PI, 4 % DMPE and about 3 % PA. To identify species (fatty acid composition) of the major phospholipids from LP, MS analyses can be performed. Fig. 2 shows an example of PC and PE species analysis from LP isolated from wild type yeast grown on YPD (glucose) or YPO (oleic acid). PC was found to contain primarily C16:1/C16:1 and C16:1/C18:1 fatty acids when cells were grown on glucose as carbon source (Fig. 2A). PC from cells grown on oleate contains mainly C18:1/C18:1 with only minor amounts of C18:1/C16:1. The PE species C16:1/C18:1 and C16:0/C18:1 or C16:1/C18:0 were predominant when cells were grown on YPD

(Fig. 2B). PE with C18:1/C18:1 was the major species when cells were cultivated on oleic acid (YPO).

FIGURE 2 here

4. Neutral lipids of LP were analyzed by TLC and identified by comparison to standard mixtures (Fig. 3A). As cholesteryl ester was used as standard for steryl esters the R_f-value is slightly higher (Fig. 3A, lanes 1 and 2) than for fatty acyl esters of ergosterol and precursors (lanes 3 and 4). The neutral lipid pattern of LP changes depending on the carbon source used for the cultivation of cells. LP from cells grown on glucose contain large amounts of STE and approximately equal amounts of TAG, whereas LP from cells grown on oleic acid as carbon source mostly accumulate this fatty acid in TAG. Other non-polar lipids are missing in wild type LP when cells are grown under standard conditions. The amounts of the different neutral lipids were quantified by densitometric scanning, and relative amounts (µg lipid/µg protein) were calculated. LP from cells grown on YPD contain approximately 47.2 % STE and 52.8 % TAG, whereas LP from cells grown on YPO have 0.2 % STE and 99.8 % TAG (our own unpublished data).

FIGURE 3 here

5. TAG species analysis of the neutral lipid fraction from cells grown on YPD or YPO, respectively, is shown in Fig. 3B. TAG from cells grown on YPD mainly contain C16:1/C16:1/C18:1 (50:3 species) and C18:1/C18:1/C16:1 (52:3 species), whereas the majority of TAG from cells grown on YPO is C18:1/C18:1/C18:1 (54:3 species). In

general, most of the yeast TAG contains monounsaturated fatty acids. Polyunsaturated acyl chains were only found in TAG from cells grown on YPO, e.g. C18:1/C18:1/C18:2 (54:4 species) due to the presence of polyunsaturated fatty acids as impurity in the oleic acid used a carbon source.

6. Isolated LP were analyzed for sterol composition as described previously (Czabany *et al.*, 2008). In brief, ergosterol was found to be the major sterol with approximately 70 % of total sterols followed by the precursors zymosterol (10 %), fecosterol (~7 %) and episterol (~7 %).
7. Fatty acid analysis of yeast neutral lipids has also been reported recently (Czabany *et al.*, 2008). It was found that TAG as well as STE contain mostly C18:1 (40-50 %) followed by C16:1 (~35 %) and only minor amounts of C16:0 and C18:0.

5. Notes

1. Delipidation of LP samples prior to TCA precipitation of proteins can be omitted, but disturbing effects during SDS-PAGE may be observed. Washing the precipitated protein pellet with cold acetone helps to circumvent this negative effect and is recommended.
2. If the purity of a LP preparation does not show the desired quality due to contamination with other cellular membranes, a further purification step can be added to the standard protocol. For this purpose, LP preparations are treated with 4.5 M urea. After 15 min of incubation at RT, the last floating centrifugation step of the standard procedure is repeated, and highly purified LP can be collected from the top of the gradient.

3. When highly concentrated LP samples are needed, one further floating centrifugation step using a table top centrifuge at maximum speed for app. 15-30 min can be performed. The excess amount of buffer under the LP layer at the top can be removed using a syringe.
4. Induction of LP proliferation with oleic acid increases the yield of LP markedly but at the same time alters the lipid composition dramatically as shown in the previous section.
5. If a yeast strain needs to be cultured on selective minimal media the yield of LP may be extremely low. Large culture volumes may be required to obtain LP at substantial quantities.
6. Neutral lipid analysis by densitometric measurement strongly depends on the intensity of the band color. Therefore, it is necessary to routinely compare bands to a standard loaded onto the same TLC plate. STE are stained more intensively than TAG by the charring method which has to be taken into account when using standards. Moreover, bands should not be too broad to avoid problems during the scanning process.

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Table 1

Marker antibodies used for the quality control of lipid particles.

LP = lipid particle; ER = endoplasmic reticulum; Mt = mitochondria; Vac = Vacuole

Marker Protein	**Subcellular Localization**	**Function**
Erg1p	LP, ER	Squalene epoxidase
Erg6p	LP, ER	Δ(24)-Sterol C-methyltransferase
Erg7p	LP, ER	Lanosterol synthase
Ayr1p	LP, ER	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase
Prc1p	Vac	Vacuolar carboxypeptidase Y
Por1p	Mt	Mitochondrial porin
Wbp1p	ER	β-Subunit of the oligosaccharyl transferase (OST) glycoprotein complex
Sec61p	ER	Integral ER required protein for protein import

Figures

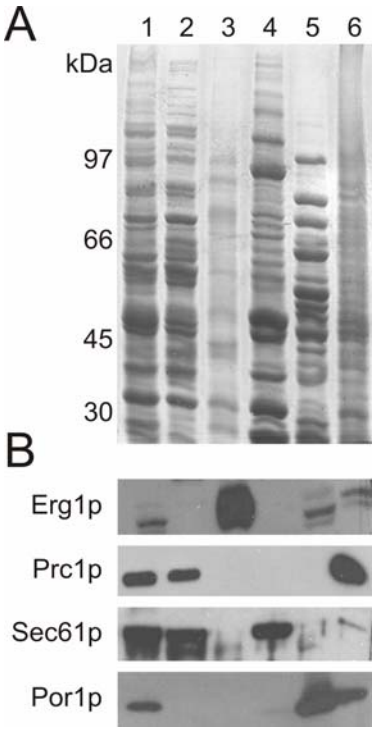

Figure 1

Quality control of lipid particles.

(A) Protein pattern of homogenate (lane 1), cytosol (lane 2), lipid particles (lane 3), endoplasmic reticulum (lane 4), mitochondria (lane 5) and vacuoles (lane 6). Proteins were separated on a 12.5 % SDS gel as described in the Methods section. (B) For Western blot analysis, 15 µg protein from each fraction were separated by electrophoresis, blotted, and probed with the respective antibodies.

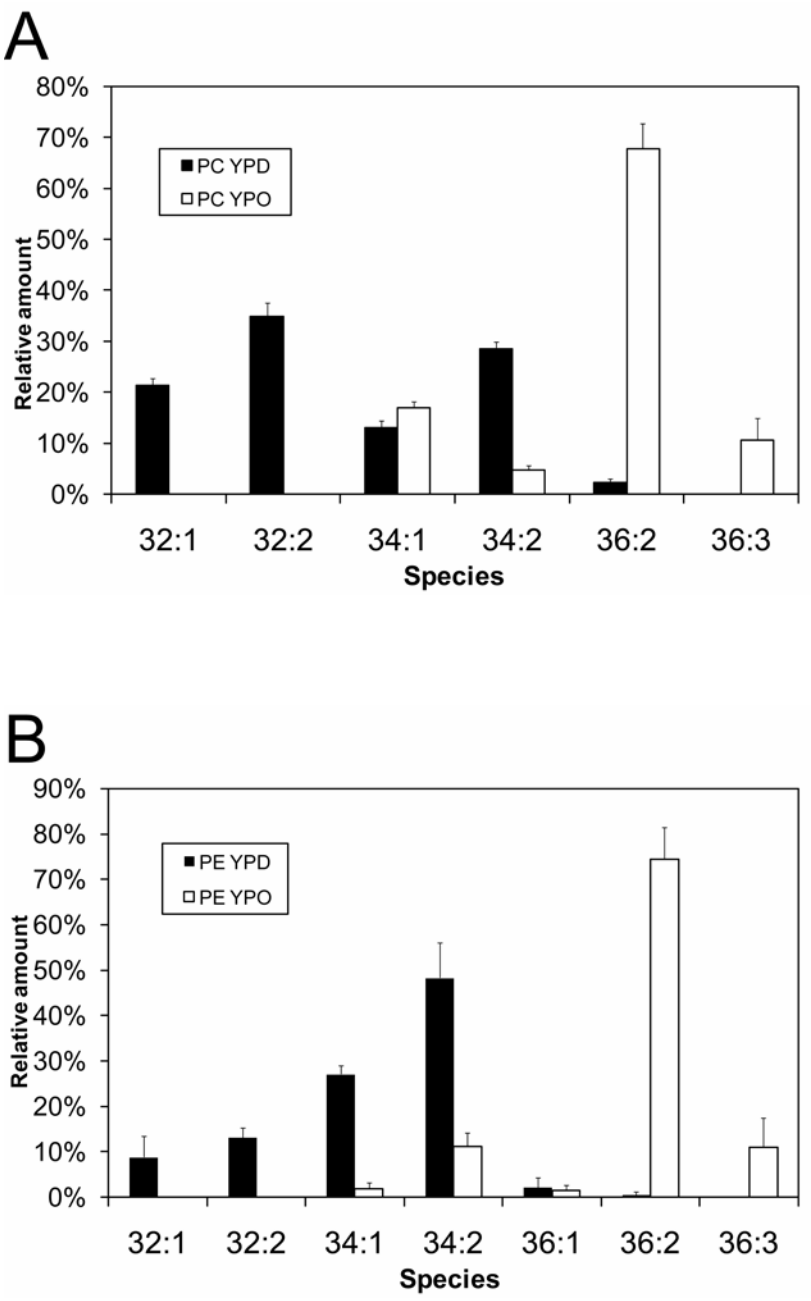

Figure 2

Mass spectrometric analysis of phospholipids from lipid particles.

Phosphatidylcholine (A) and phosphatidylethanolamine (B) species were analyzed by MS as described in the Methods section. LP were isolated from cells grown on YPD (black bars) and YPO (white bars). Species are formed as follows: 32:1 (16:1/16:0); 32:2 (16:1/16:1); 34:1 (16:1/18:0) or (16:0/18:1); 34:2 (16:1/18:1); 36:2 (18:1/18:1); 36:3 (18:1/18:2) or (18:0/18:3).

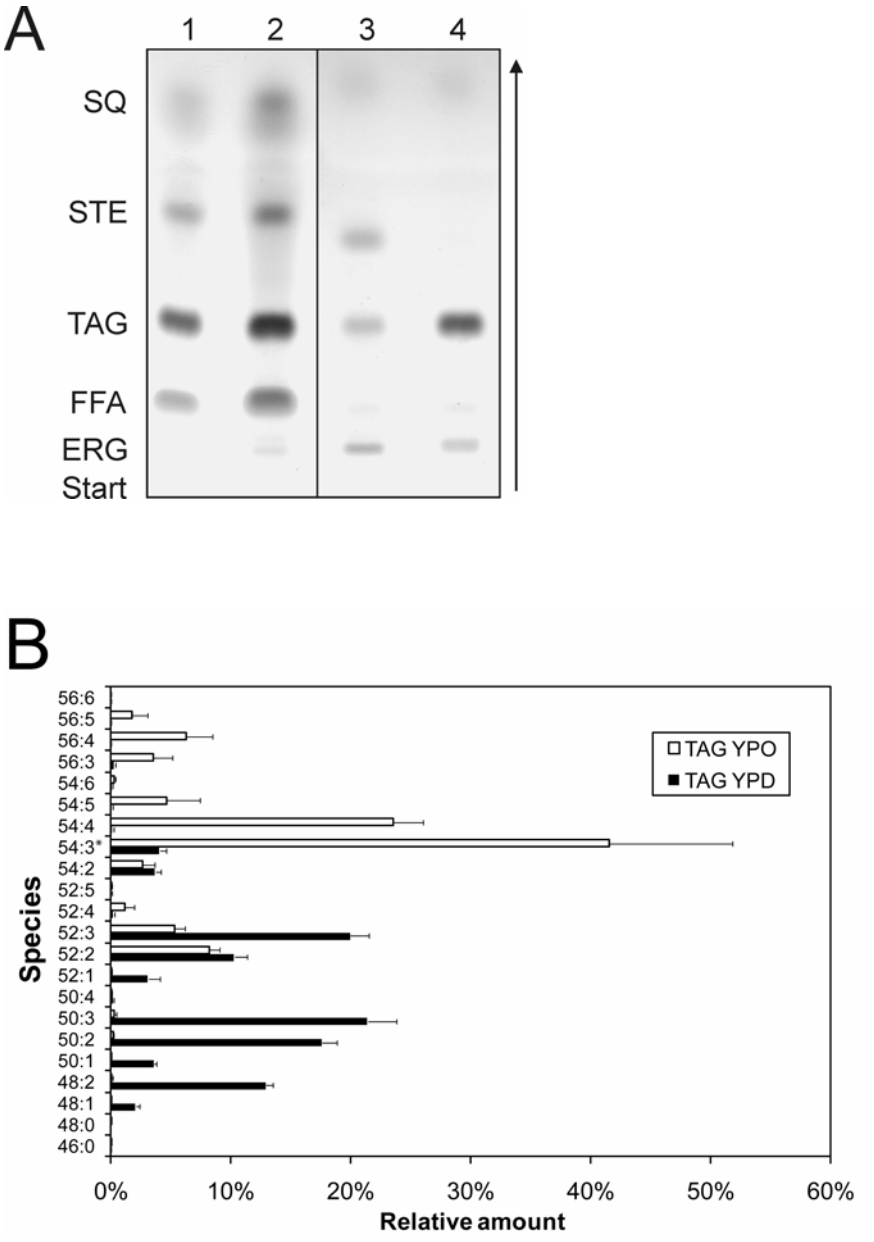

Figure 3

Neutral lipids of lipid particles from wild type yeast cells grown on different carbon sources.

(A) Neutral lipids were separated by TLC in a two-step procedure using light petroleum/diethyl ether/acetic acid (70:30:2; per vol.) as the first solvent approximately to one half of the distance on the plate, and light petroleum/diethyl ether (49:1; v/v) as the second

solvent (see Methods section). The arrow indicates the ascending mode of TLC. Different neutral lipid classes are indicated at the left. SQ: squalene; STE: steryl esters; TAG: triacylglycerols; FFA: free fatty acids; ERG: ergosterol. 1 and 2: standard mixtures containing the different neutral lipid classes; 3: lipid pattern of LP from cells grown on glucose; 4: lipid pattern of LP from cells grown on oleic acid. (B) Mass spectrometric analysis of TAG from LP of cells grown on YPD (black bars) or YPO (white bars), respectively. The relative amount of the signal for the different species is shown. The asterisk indicates three C18:1 chains in a TAG molecule.

Chapter 5

Lipid particles of the yeast *Saccharomyces cerevisiae*

Lipidome meets Proteome

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Key words: triacylglycerol, phospholipid, mass spectrometry, protein, lipid particles, yeast

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Abbreviations: TAG: triacylglycerols; STE: steryl esters; LP: lipid particles

Abstract

In the yeast *Saccharomyces cerevisiae* as in other eukaryotes neutral lipids are a reservoir of energy and building blocks for membrane lipid synthesis. The yeast neutral lipids, triacylglycerols (TAG) and steryl esters (STE), are stored in so called lipid particles (LP) as biologically inert form of fatty acids and sterols. Prerequisite for the understanding of LP function and structure is elucidation of their molecular equipment. For this purpose, we performed conventional analysis and mass spectrometric analysis of lipids (TAG, STE, phospholipids), and MS analysis of proteins which are present on the surface of LP. These analyses were carried out with LP from cells grown on the two carbon sources glucose and oleic acid. Results obtained by these methods revealed marked differences in the lipidome, but also in the proteome of LP isolated from yeast cells grown under different conditions. Changes in the cultivation conditions toward oleic acid led to serious intracellular adaptations regarding the lipid composition of neutral lipids and glycerophospholipids. Most notably we found a huge increase in the amount of TAG at the expense of STE with oleic acid being the major fatty acid esterified in TAG. Levels of phospholipids were also strongly influenced by the change of carbon source, with each of the phospholipids responding in a case sui generis. Apparently, the shift of the carbon source caused a strong increase in the degree of unsaturation with a strong tendency toward the mono-unsaturated oleic acid. Moreover, proteome analysis of LP led to identification of several new putative LP proteins. The detailed analysis of the lipid and protein composition of this organelle will help to deepen our knowledge regarding function of LP in the cellular interplay of organelles.

Introduction

A strong increase of adipositas and obesity mainly related to unbalanced diet led scientists to study of lipid uptake, biosynthesis, storage and mobilization with high priority. Lipid (fat) metabolism and especially storage are inevitably linked to one specific organelle named the lipid particle (LP), lipid droplet or oil body. The baker's yeast *Saccharomyces cerevisiae* has a long standing tradition in lipid particle research. Using this experimental system fundamental studies addressing structural, functional and metabolic aspects of lipid particles were performed (Clausen *et al.*, 1974; Zinser *et al.*, 1993; Leber *et al.*, 1994; Czabany *et al.*, 2008). One characteristic property of LP is the surface monolayer of phospholipids which protects the highly hydrophobic interior from the cellular environment (Tauchi-Sato *et al.*, 2002). The polar head groups of these phospholipids face the cytosol, and the hydrophobic side chains (fatty acids) associate with the non-polar core of the LP. This hydrophobic core which represents more than 95 mass % of LP consists of the non-polar lipids triacylglycerols (TAG) and steryl esters (STE) which lack charged groups and are therefore not suited as constituents of membrane bilayers. As shown recently in our laboratory, STE of LP form several ordered shells below the phospholipid monolayer, whereas TAG are more or less randomly packed in the center of LP (Czabany *et al.*, 2008).

The surface phospholipid monolayer membrane of LP contains a small but specific set of proteins embedded. Prominent proteins of mammalian LP are perilipin, adipophilin, hormone-sensitive lipase (HSL), adipose TAG lipase (ATGL), TIP47 (Tail-Interacting Protein 47 kDa), PAT-proteins, S3-12 and OXPAT (Goodman, 2008; Olofsson *et al.*, 2008; Thiele and Spandl, 2008; Farese, Jr. and Walther, 2009; Goodman, 2009; Bickel *et al.*, 2009; Robenek *et al.*, 2009; Yamaguchi and Osumi, 2009; Ohsaki *et al.*, 2009). A functional link between mammalian lipid droplets to the spliceosome and proteasome was suggested (Ohsaki

et al., 2006; Cho *et al.*, 2007; Guo *et al.*, 2008). The most prominent proteins from plant oil droplets are the oleosins which cover the surface of the droplet and prevent them from coalescence (Huang *et al.*, 2009). In yeast LP, so far homologues of perilipins or oleosines were not detected. However, a number of proteins involved in lipid metabolism are characteristic for this organelle (Athenstaedt *et al.*, 1999; Rajakumari *et al.*, 2008). Besides storing lipids as chemical energy and building blocks of biological membranes, yeast LP were assumed to be important players of lipid homeostasis (Kurat *et al.*, 2009). The mechanism by which LP proteins are targeted to and associated with the LP surface is still a matter of dispute. A favoured model suggests that TAG and/or STE synthesizing enzymes form large amounts of non-polar lipids between the two leaflets of the ER which finally results in budding of a nascent LP (Athenstaedt and Daum, 2006; Czabany *et al.*, 2007; Rajakumari *et al.*, 2008; Walther and Farese, Jr., 2009; Ohsaki *et al.*, 2009). Some alternative mechanisms have also been discussed (Robenek *et al.*, 2006) but all of them agreed that LP evolve from the ER.

The composition of LP strongly depends on the carbon source used for cell cultivation (Athenstaedt *et al.*, 2006). A helpful tool for making yeast cells “obese” is growth on oleic acid. Under these conditions, peroxisome proliferation is induced because this organelle is the only subcellular fraction in yeast where β-oxidation of fatty acids occurs (Erdmann *et al.*, 1989). Nevertheless, oleic acid grown yeast cells accumulate large amounts of lipids with a substantial portion of oleic acid (Rosenberger *et al.*, 2009). Moreover, fatty acids may act as signalling molecules in gene expression. As an example, oleic acid binds to upstream promoter elements with ORE (oleate response element) being the most prominent (Duplus *et al.*, 2000; Trotter, 2001).

The aim of the present study was to re-visit the yeast LP and to explore molecular details of the molecular composition of this subcellular compartment. Knowledge about lipid

and protein components forming LP is an important prerequisite for a broader understanding of its function. Highly improved and sophisticated methods of mass spectrometry were employed to analyse the lipidome of LP on one hand and the proteome on the other hand. Variation of the carbon source (glucose vs. oleate) for growth of yeast cells allowed us to isolate variants of LP which strongly differed in their lipid composition. Here we describe how these changes affected not only the lipidome but also the proteome of LP thereby highlighting general as well as adaptative aspects of LP formation.

Methods and Materials

Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. Cells were grown at 30°C in rich medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD) or 0.3% yeast extract, 0.5% peptone, 0.1% glucose, 0.5% KH_2PO_4 and 0.1% oleic acid (YPO). For solubilizing fatty acids in YPO, 0.2% Tween 80 was added to the media.

Subcellular fractionation of yeast cells

Subcellular fractions of yeast cells were prepared by published procedures (Serrano, 1988; Leber *et al.*, 1994; Zinser and Daum, 1995) and routinely tested by Western Blot analysis (Haid and Suissa, 1983) using rabbit antibodies against Erg1p, Por1p, Pma1p, Pcs60p and Prc1p/CPY. Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Protein analysis

Proteins from isolated LP and homogenates were precipitated with trichloroacetic acid (TCA) at a final concentration of 10%. Proteins were quantified by the method of Lowry *et al*. (Lowry *et al.*, 1951) using bovine serum albumin as standard. Prior to protein analysis, samples of LP were delipidated. Non-polar lipids were extracted with two volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were analyzed as described above. SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was carried out as described by Laemmli (Laemmli, 1970) using 12.5% separation gels.

Lipid analysis

Lipids from yeast cells grown on YPD or YPO were extracted as described by Folch *et al*. (Folch *et al.*, 1957). For quantification of neutral lipids extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4), and chromatograms were developed in an ascending manner by using the solvent system light petroleum/diethyl ether/acetic acid (25:25:1; per vol.) for the first third of the distance. Then, plates were briefly dried and further developed to the top of the plate using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of ergosteryl esters was carried out by densitometric scanning at 275 nm with a Shimadzu dual wavelength chromatoscanner CS-930 using ergosterol as standard. To further analyze TAG, TLC plates were dipped into a charring solution consisting of 0.63 g of $MnCl_2 \cdot 4H_2O$, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulphuric acid, briefly dried and heated at 100 °C for 30 min. Then, lipids were quantified by densitometric scanning at 400 nm using a Shimadzu dual-wave length chromatoscanner CS-930 with triolein as standard.

For phospholipid analysis, lipid extracts from homogenate and lipid particles were loaded manually on silica gel 60 plates (Merck, Darmstadt, Germany). Individual phospholipids were separated by two dimensional TLC using chloroform/methanol/25% NH_3 (65:35:5; per vol.) as first, and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) as second developing solvent. Lipids were stained with iodine vapour, scraped off the plate and quantified by the method of Broekhyuse (Broekhuyse, 1968). For quantification of total phospholipids the respective band from neutral lipid analysis (see above) was scraped off the plate and analyzed by the method of Broekhuyse (Broekhuyse, 1968).

Mass spectrometry of neutral lipids and phospholipids

Lipid extracts were prepared as described above and diluted 1:100 in acetonitrile/2-propanol (5:2; v/v), 1% ammonium acetate, 0.1% formic acid. As internal standards 5 µM TAG (species 51:0) and PC (species 24:0) were added. For chromatographic separation a thermo hypersil GOLD C18 column (100 x 1 mm, 1.9 mm) was used employing solvent A (water with 1% ammonium acetate, 0.1% formic acid) and solvent B (acetonitrile/2-propanol (5:2; v/v);1% ammonium acetate; 0.1% formic acid). Gradients were established from 35 to 70 % B for 4 min and then to 100% B in another step of 16 min. These conditions were held for 10 min with a flow rate of 250 ml/min. Mass spectrometry was performed by HPLC direct coupling to a FT-ICR-MS hybrid mass spectrometer (LTQ-FT, Thermo Scientific) equipped with an IonMax ESI source. The mass spectrometer was operated at an accuracy of <2 ppm with external calibration and a resolution of 200,000 full width at half height (FWHH) at m/z 400. The spray voltage was set to 5,000 V, capillary voltage to 35 V, and the tube lens was at 120 V. The capillary temperature was at 250 °C. Peak areas are calculated by QuanBrowser for all lipid species identified previously by exact mass (<2 ppm) and retention time.

Calculated peak areas for each species were expressed as % of the sum of all peak areas in the respective lipid class.

Mass spectrometry of proteins

Digestion: After TCA precipitation, 100 µg of the protein pellet was dissolved in 1 ml of 25 mM NH_4HCO_3 in an Eppendorf tube. Disulfide bridges were reduced in 45 mM DTT for 1 h at 60°C and 400 rpm shaking in a Thermomixer comfort (Eppendorf). The solution was then allowed to cool to room temperature, and cysteine residues were alkylated in the presence of 100 mM iodoacetamide for 45 min in a dark at room temperature. To avoid subsequent alkylation of trypsin, the reaction was quenched after 45 min by adding additional 12.5 µl of 45 mM DTT and incubating for another 45 min at room temperature. Then, trypsin was added to the reduced and alkylated samples to obtain a protein/enzyme ratio of 1:50 (w/w). The solution was incubated overnight at 37 °C. The digestion was stopped by addition of 1 µl of a 10 % trifluoroacetic acid (TFA) solution.

HPLC separation: Prior to nano-liquid chromatographic (nLC) separation, the samples were vacuum concentrated to approximately 8 µl and Solvent A (8% ACN, 0.1% TFA) was added to a final volume of 15 µl. Separation was performed on a Proxeon Biosystems EASY-nLC™ system (Odense, Denmark) coupled to a SunCollect MALDI spotting device (Sunchrom, Germany). The peptides were loaded onto an in-house packed 100 µm x 30 mm pre-column (Waters X-Bridge™ BEH 180 C_{18} 300 Å 3.5 µm,) and desalted with 30 µl solvent A for 15 min and separated on an in-house packed 100 µm x 150 mm column (Waters X-Bridge™ BEH 180 C_{18} 300 Å 3.5 µm) at a flow rate of 400 nl/min. The gradient profile linearly increased from 8 to 45% solvent B (92% ACN, 0.1% TFA) within 100 min, to 90% B within 20 min, 10 min at 90% B, back to 8% B within 5 min and at 8% for another 5 min. The

effluent from the LC was pre-mixed with matrix, α-cyano-4-hydroxycinnamic acid, via a tee (Upchurch Scientific, USA) from an auxiliary pump (flow rate, 1.2 µl/min) and spotted every 20 sec on a blank 123 x 81 mm Opti-TOF™ LC/MALDI Insert metal target. This matrix solution contained 3.5 mg/ml α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) dissolved in 70% ACN, 30% H_2O, 0.1% TFA, spiked with 60 fmol [Glu^1]-Fibrinopeptide B (Bachem, Weil, Germany) for internal calibration.

MS and MS/MS: Mass spectra were acquired using an Applied Biosystems/MDS Sciex 4800 TOF/TOF™ Analyzer. The instrument was equipped with a Nd:YAG laser, emitting at 355 nm with a repetition of 200 Hz. All spectra were acquired in the positive reflector mode between 700 and 4,500 *m/z* with fixed laser intensity. A total of 750 laser shots per spot were accumulated. An 8-point plate model External calibration was performed using a Sequazyme™ Peptide Mass Standards Kit (Applied Biosystems). Fragmentation was performed with collision energy of 1 kV using air as collision gas at a pressure of 1 x 10^{-6} Torr. To reduce sample consumption during measurement, stop conditions for MS/MS were defined. A minimal number of 15 peaks above 45 S/N with at least 12 accumulated sub-spectra; a minimum of 1,250 and maximum of 2,500 laser shots were recorded. To avoid unnecessary multiple selections of identical precursor, MS/MS precursor selection was carried out via the instrument's software, job-wide interpretation. A total of 6 precursors per spot with a minimum signal-to-noise-ratio of 80 were selected for fragmentation. Potential matrix signals were removed from precursor selection, by excluding all masses in the range from 700 to 1,400 *m/z* having values of .030 +/- 0 .1 *m/z* as well as internal calibration.

Database searches and interpretation: Mascot Generic Format (MGF) files were retrieved from each MALDI MS/MS spectra using the built-in Peaks2Mascot feature, exporting up to 65 peaks per MS/MS spectrum, each requiring a minimum signal-to-noise of 5. The MGF files were processed using the Mascot™ database search engine v2.2.03 (Matrix

Science Ltd., UK). The following settings were used: Enzyme, trypsin; allowed miss cleavages for trypsin 3, fixed modification, carboxymethylation of cysteine; variable modification, oxidation of methionine. MS precursor mass tolerance was set to 50 ppm and MS/MS mass tolerance to 0.5 Da. The search was performed with a custom Saccharomyces Genome Database generated from Saccharomyces Genome Database (http://www.yeastgenome.org) containing 6,717 entries as at November 30th 2008. A decoy database consisting of same-length random protein sequences was automatically generated and searched. All statistical analyses were based on peptides having Mascot™ MS/MS ions scores exceeding the "identity or extensive homology threshold" ($p < 0.05$). In the case of multiple fragmentations of identical precursors, due to recurrence in repetitive runs, only data from the highest scoring peptide were kept.

Results

Exogenous oleic acid is preferentially incorporated into triacylglycerols

Growth of yeast cells on oleic acid dramatically affects the lipid metabolism and has a major impact on the lipid storage organelle, the lipid particle (LP). Whereas wild type cells cultivated on glucose (YPD) contain roughly same amounts of storage lipids TAG and STE, growth on oleate dramatically increased the amount of TAG in LP at the expense of STE (Table 2). To address the massive formation of TAG in more detail, molecular species of this lipid class from LP were analyzed by mass spectrometry. TAG patterns from cells grown on YPD or YPO, respectively, showed marked differences (Figure 1). When cells were grown on glucose, 52:1, 52:2, 52:3 and 54:2 constituted the major TAG species. The 52 species contain one C16 and two C18 fatty acids. Either one, two or all three fatty acids can be unsaturated.

Stereospecific positions could not be deduced from this analysis. The four TAG species mentioned above displayed approximately 65% of total TAG. The remaining species contained two or three C16 fatty acids, either saturated or unsaturated. Approximately 5 % of 54:3 TAG was detected indicating that oleic acid was the exclusive fatty acid in these lipids.

When exogenous oleic acid was supplied to the media as carbon source the fatty acid pattern of the TAG changed tremendously. In contrast to cells grown on glucose, C16 was incorporated into TAG only at a minor percentage when oleic acid was the carbon source. The vast majority of fatty acids in TAG from cells grown on YPO were C18, preferentially C18:1. This result indicated that oleic acid was not only used as a carbon source, but also directly incorporated into complex lipids. Noteworthy, several TAG species containing polyunsaturated fatty acids, e.g. 54:5 species (18:1/18:2/18:2), and species with longer acyl chains, e.g. 56:3 species (C18:1/C18:2/C20:0) were detected in cells grown on YPO. This finding is due to impurities of oleate samples used as carbon source.

Growth of yeast cells on oleate increases the amount of total phospholipids

To investigate the effect of oleate as carbon source on the level and the composition of phospholipids from total cell extracts and LP, respectively, we performed conventional phospholipid analysis and mass spectrometry. As can be seen from Table 2 cultivation of cells on oleic acid resulted in an 1.5 fold increase of total phospholipids in the homogenate. An even higher increase of total phospholipids was seen with LP from YPO grown cells.

To investigate variations in distribution and abundance of individual phospholipids we subjected homogenate and LP from wild type to glycerophospholipid analysis (Table 3). In glucose grown cells, the major phospholipids from the homogenate were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) with PC being the most abundant. These major phospholipids constituted approximately 90 % of total cellular

phospholipids. Only minor amounts of phosphatidic acid (PA), phosphatidylserine (PS) and cardiolipin (CL) were detected. Homogenates from cells grown on oleate showed the same tendency with slight variations. Again, PC, PE and PI were the major phospholipids with even higher amounts of PC. The level of PE decreased for 3.5 %, whereas PI increased for 2.5 %. The amounts of PS and CL did not change much, but PA dropped to levels close to detection limits.

In LP from cells grown on YPD, PC, PE and PI were also the most prominent glycerophospholipids. Interestingly, levels of PC and especially PI were higher and that of PE lower in LP than in the homogenate. Other phospholipids occurred only at negligible amounts in LP. When cells were grown on oleate, the phospholipid pattern of LP was very similar to that of cells grown on YPD. The only marked exception was PS which dropped from ~2 % on YPD to ~0.8 % on YPO.

Mass spectrometric analysis of phospholipids from homogenates and lipid particles

As described above no major differences were observed in the pattern phospholipid classes of LP from cells grown on glucose and oleate. These experiments, however, did not include species analysis. Therefore, we extended our studies to mass spectrometry of phospholipids from homogenates and lipid particles. The fact that major fatty acids of the yeast *Saccharomyces cerevisiae* are of the C16 and C18 types makes the species patterns rather simple. Therefore, as expected, PC, PE, PS and PI from cells grown on glucose showed a strong preference for C16 and C18 in their mono-unsaturated or saturated form (Figure 2). Nevertheless, the species patterns of individual phospholipids varied in a most typical way. PE showed a strong preference for 34:2 species (C16:1/C18:1), whereas in PC 32:2 and 34:1 occurred at a similar level. Only small amounts of 36:2 (C18:1/C18:1) were detected in PE and PC. Contrary to PE and PC, PI and PS showed a strong preference for 34:1 species. In PI

the second abundant species was 32:1, whereas in PS 34:2 occurred as the other major species. Another interesting observation was the presence of substantial amounts of 36:1 in PI. This species was also found in PC, but not in PS and PE. PS also contained substantial amounts of 28:0 which is most likely composed of C16:0 and C12:0.

Mass spectrometry of phospholipids from homogenates of cells grown on oleate showed a completly different distribution of fatty acyl species. Not surprisingly, the incorporation of oleic acid (C18:1) into phospholipids was high under these cultivation conditions. The major phospholipids behaved more or less in pairs. PC and PE strongly preferred oleic acid as a constituent (see Figure 2) resulting in a strong appearance of 36:2 species. This effect was most pronounced in PC, whereas in PE C18:1 was combined with C16:1 (34:2 species). Species patterns of PI and PS from cells grown on oleate were different. Although in both phospholipids oleic acid was highly present in the di-unsaturated species 36:2, the 34:1 species (C16:0/C18:1 or C16:1/C18:0) occurred at an almost equal level. In PS and PE 34:2 was another prominent combination of fatty acids. The appearance of 36:3 species in all phospholipids was due the impurities of oleate samples used a carbon source as described above.

When phospholipid species from LP of cells grown on glucose (Figure 3) were analyzed we realized that in most cases the species patterns reflected very much those of phospholipids from total cell extracts (see Figure 2) following the equilibrium rule “You take what you get”. However, one major exception was the large amount of 32:2 PE in LP which did not occur in the homogenate. Additionally, the two fully saturated species 34:0 (C16:0/C18:0) and 36:0 (C18:0/C18:0) appeared in PS from the LP fraction, which were obviously below the detection limits in the total cell extract.

Differences between the phospholipid species patterns of LP and homogenate from YPO grown cells were mainly in the PI and PS fractions. Whereas PE and PC from LP

exhibited a similar species pattern as the homogenate, the amount of 34:1 PI in LP was increased at the expense of 36:2 PI, and the level of 36:2 PS in LP was much higher than in homogenate. However, despite the strong influence of exogenous oleic acid on the formation of phospholipid species, the individual phospholipid classes retained at least some typical constituents.

Whereas species analysis of phospholipids yields a precise information of molecular components, the ratio of saturated to unsaturated species gives us an overview of properties which may be important for the physical status of membranes formed from different phospholipid species. It is remarkable that the different phospholipid classes exhibited marked differences in their degree of saturation (Table 4). As the most striking example, PI is the phospholipid containing most saturated fatty acids followed by PS irrespective of the carbon source used for the cultivation of cells. In LP from cells grown on glucose a slight selectivity seems to occur insofar as more unsaturated PE and PC species accumulate compared to the homogenate. Oleate as a carbon source led to a more than 3 fold reduction of saturated PE and PC species, but only to a moderate although pronounced effect on PI and PS. Also in oleate grown cells, LP attracted more unsaturated PE and PC species than bulk membranes, but this effect was also observed with PS. In contrast, the level of saturated PI species in LP remained high even when oleate was present as carbon source.

Influence of the carbon source on the pattern of lipid particle proteins

During the last decade, the yeast LP proteome was studied based on mass spectrometry and localization of GFP-tagged proteins (http://www.yeastgenome.org/12182009; http://yeastgfp.yeastgenome.org/12182009). Currently, 41 LP proteins were identified by these criteria (Table 5). Interestingly, most of these proteins are not exclusively localized to the LP but seem to have a dual localization with

additional occurrence in microsomal and mitochondrial fractions. Previous experiments (our own unpublished data) had led us to the assumption that the existing list of LP proteins is not complete. We assumed that due to methodological limitations LP proteins of low abundance escaped identification so far. Therefore, we re-addressed this issue by employing a novel proteomic method of nano-LC-MS (see Methods section). Moreover, we tested whether change of growth conditions (YPD vs. YPO) had an impact on the LP protein pattern.

Protein samples were generated from LP originating from cells that were either grown on glucose or oleic acid as the carbon source. An initial analysis for these samples by SDS-Page (Fig. 4A) revealed already qualitative differences between the two LP variants. Both LP preparations were of high purity (Fig. 4B) which was important for the further proteome analysis. A primary survey obtained data by NanoLC-MS is shown in Figure 5. With LP from cells grown on glucose 49 proteins were identified; 22 of these polypeptides had already been assigned to LP. Analysis of LP prepared from oleic acid grown cells resulted in the identification of 54 proteins; 24 of these proteins were already known as LP constituents. All proteins identified from glucose grown cells were also found with oleic acid as carbon source. It is tempting to speculate that these 20 proteins are the most abundant and standard proteins of LP.

In addition to the known set of LP proteins some other polypeptides were identified as novel although putative LP proteins. Surprisingly, only 6 proteins (see Table 5, proteins marked with §) that were so far not assigned to LP were identified on both variants (YPD and YPO) of LP, namely Cpr5p, Gtt1p, Osh4p, Ubx2p, Vps66p and Ypt7p. In data bases, these proteins had been assigned to other subcellular compartments, mainly the cytosol and the ER. Two aspects have to be taken into account for further and more precise localization of these proteins. First, we have to be aware of the dual localization of LP proteins as shown before which made biochemical and microscopic analysis difficult. Since LP are believed to

originate from the ER (Rajakumari *et al.*, 2008) such localization scenarios may be expected. Secondly, of course, contamination of LP with other subcellular compartments has to be considered. This problem has to be ruled out by precise molecular investigations. So far, proteins identified by this proteomic approach were not only assigned to LP, but also to the ER, mitochondria, cytosol and plasma membrane (Figure 6). Comparing localization patterns of the newly found proteins revealed that small variations can be seen depending on the carbon source used for cultivation. Interestingly, the percentage of potential ER resided proteins was strongly reduced when LP from oleic acid grown cells were investigated, whereas the portion of proteins assigned to the cytosol was doubled. Notably, several ribosomal and nuclear proteins were found associated with LP when cells were grown on oleic acid.

Another interesting aspect is function of proven and putative LP proteins. A large number of LP proteins are involved in lipid metabolism both from YPD and YPO grown cells (Figure 7). Other functions attributed to these proteins were protein glycosylation, cell wall organization or ER unfolded protein response. For a remarkably large number of proteins biological functions have not been annotated. Several clusters of proteins appear to be strongly affected by the change of carbon source. As examples, proteins involved in ER unfolded protein response were only found in LP from cells grown on YPD, and the big portion of proteins involved in protein glycosylation was drastically reduced in cells grown on YPO. In contrast, a large number of proteins involved in translation and energy providing processes were found in LP from cells cultivated on oleic acid.

Discussion

Lipid particles (lipid droplets, oil bodies) have gained much attention during the last decade due to their role as lipid buffer and involvement in human diseases and plant oil storage. Moreover, we are at the beginning to understand the metabolic functions that might be governed by LP. For all these reasons knowledge about structure and molecular equipment of LP is required. We are using the yeast *Saccharomyces cerevisiae* as a model for such studies because of the parallel features to other experimental systems and the convenience of genetic and nutritional manipulation of this microorganism.

Lipid particle lipidome

In the past, several attempts were made to obtain an overview of the lipid pattern of LP (Zinser *et al.*, 1993; Leber *et al.*, 1994; Schneiter *et al.*, 1999). These studies were mainly meant to explain lipid storage in this organelle. More recently, the technology of mass spectrometry enabled us to study lipid components of LP in molecular detail (Connerth *et al.*, 2009). These methods are based on systematic lipidome analyses of yeast lipids as published by Ejsing *et al.*(Ejsing *et al.*, 2009) and Xue Li Guan and Wenk (Guan and Wenk, 2006).

Recent studies from our laboratory (Rosenberger *et al.*, 2009) Connerth et al., manuscript in preparation) and by others (Garbarino and Sturley, 2009; Petschnigg *et al.*, 2009) showed that growth of yeast cells on oleate (YPO medium) greatly stimulated the proliferation of yeast LP. Under these conditions, the requirement for fatty acid storage increases and makes LP to a more prominent cell organelle. Hence, we decided to expand our studies of LP component analysis to variation of culture conditions, namely growth of cells on glucose or oleic acid.

Two observations made with TAG from cells grown on glucose were interesting. First, there seems to be some selectivity regarding the fatty acid pattern of TAG (see Fig. 1) compared to total lipids insofar as TAG contain more C18:1 (oleic acid) than bulk lipids. The reason for this finding may be preference of the TAG synthesizing enzymes Dga1p and Lro1p (Oelkers *et al.*, 2002; Czabany *et al.*, 2007) for the respective substrates. Secondly, and result of the first observation was that a large portion of TAG contains unsaturated fatty acids. This portion of storage lipids appears to have a rather high degree of fluidity. The most obvious effect of growing cells on YPO was the huge increase in the amount of TAG, whereas the amounts of STE decreased dramatically close to detection limits. We (Connerth *et al.*, manuscript in preparation) showed recently that neither a transcriptional regulation of the STE synthesizing enzymes Are1p and Are2p nor any posttranslational modification was the reason for the depletion of STE. We demonstrated that free oleic acid directly inhibits STE synthases. Interestingly, oleic acid had the opposite effect on the pattern of non-polar lipids in another yeast. Athenstaedt *et al.* (Athenstaedt *et al.*, 2006) observed in the yeast *Yarrowia lipolytica* grown on oleate a decrease of TAG but more STE compared to LP from glucose-grown cells. The other effect of oleate as a carbon source for *Saccharomyces cerevisiae* was that this fatty acid was obviously not only degraded by β-oxidation in peroxisomes under these conditions (Hiltunen *et al.*, 2003) but also directly incorporated as building block of complex lipids. This effect led to large amounts of oleic acid in TAG.

Although phospholipids are only a minor component of LP they may play an important role of this organelle. First, phospholipids of the LP surface monolayer determine the contact of the highly hydrophobic particle to the hydrophilic environment. Therefore, an appropriate shielding is required. Second, the small set of proteins embedded in the surface phospholipid monolayer of LP may need a specific membrane environment for functionality. Since we do not know details about the enzymology of LP proteins we can only speculate on

this point. Finally, the surface membrane lipid composition is most likely result of the biogenesis process of LP. If we accept budding of LP from the ER as a realistic model for LP biosynthesis, at least large parts of the LP surface membrane have their origin in the ER. A combined selectivity for phospholipids and proteins (see below) may be anticipated.

The question arises whether or not there is something specific with LP phospholipids. As can be seen from Table 3, PI seems to be significantly enriched in LP, mainly at the expense of PS and PE. Given the fact that PI and PS belong to the group of negatively charged phospholipids, a replacement would most likely not lead to dramatic effects. However, the decrease of PE which is known as a bilayer disturbing phospholipid appears to make sense in the monolayer membrane, where such an effect may become superfluous. Whether or not the enrichment of 32:2 PE species in LP has a specific effect remains open. Enrichment of PI on the LP surface may increase the charge in the monolayer. It is known that inositol phospholipids including PI are also important for interfacial binding of proteins and regulating proteins at the cell interface. As PI is polyanionic, it can be very effective in creating unspecific electrostatic interactions with proteins (Shields and Arvan, 1999; Gardocki *et al.*, 2005). The other remarkable feature of PI in total cell extracts and LP is its high degree of saturation (see Table 4). In both fractions only minor amounts of double unsaturated PI species were detected. Specific requirements for PI may explain its stable amount and composition. Interestingly, PS exhibits a similar degree of saturation as PI. Biosynthesis of both phospholipids starts with the same precursor, CDP-DAG, catalyzed by two different enzymes although with similar substrate preference (Carman and Henry, 1999). During decarboxylation of PS by Psd1p and Psd2p some selectivity is introduced (Schuiki and Daum, 2009). It is known that PSDs exhibit a clear preference regarding the molecular species of their substrate (Burgermeister *et al.*, 2004) resulting in a higher degree of unsaturation of the product which is PE. A further step of selectivity is the stepwise

methylation of PE which leads to the final product of the aminoglycerophospholipid pathway, PC. It has to be taken into account, however, the PE an PC are also formed through the CDP-Etn and CDP-Cho branches of the Kennedy Pathway with a different substrate usage and specificity (Kennedy and Weiss, 1956). Recently, a further pathway of PE formation involving the lyso-PE acyltransferase Slc4p was found (Riekhof *et al.*, 2007; Jain *et al.*, 2007) which may also contribute to the composition of the total cellular PE pool.

When yeast cells were grown on oleate, the total amount of phospholipids (see Table 2) was markedly increased. This increase was observed both with homogenate and the LP fraction. Interestingly, in the homogenate levels of PI and PC increased at the expense of PE. A similar effect was described for cells that showed a defect in unsaturation of fatty acids in an *rsp5* mutant (Kaliszewski *et al.*, 2008). Rsp5p strongly influenced the regulation of unsaturated fatty acid biosynthesis by governing the activation of two transcriptional activators Spt23p and Mga2p which in turn up-regulate the expression of the *OLE1* gene. In *rsp5-19* representing a low oleate condition the TAG level decreased whereas overproduction of the activator (high oleate conditions) led to an increase of TAG (Kaliszewski *et al.*, 2008). Neither a mutation in *RSP5* nor overproduction of the two activators mentioned above affected the level of STE.

Proteome of yeast lipid particles

The existing yeast LP proteome contained a small but specific set of 41 proteins (Athenstaedt *et al.*, 1999; Zweytick *et al.*, 2000). These proteins are embedded in the phospholipid monolayer of the lipid droplet. To our recent knowledge, yeast LP do not contain typical organelle proteins such as oleosins in plants (Capuano *et al.*, 2007) or perilipins in mammalian cellls (Greenberg *et al.*, 1991; Greenberg *et al.*, 1993). The number

of LP in different cells types is steadily increasing as methods employed get improved. As a recent example, Bartz R. *et al.* (Bartz *et al.*, 2007) expanded the proteome of mammalian lipid droplets by 70 further proteins.

The proteome approach with yeast LP presented here was aimed at the extension of the number of LP proteins but also to compare yeast LP proteins in cells growth under different conditions, namely on glucose or oleic acid. Oleic acid is the classicals substrate to induce peroxisomes in *Saccharomyces cerevisiae*, (Smith *et al.*, 2002; Gurvitz and Rottensteiner, 2006; Smith *et al.*, 2007; Saleem *et al.*, 2008). However, oleic acid also leads to massive proliferation of LP due to the huge production of non-polar lipids, especially TAG, as described also in this study. The dramatic changes in the yeast LP lipidome of cells grown on oleate led us to speculate that also the LP proteome may be affected. Differencs in LP proteins in lipid variants of this organelle have been shown before by Czabany *et al*. (Czabany *et al.*, 2008).

Interesting questions rising from this study were: (i) Do LP from YPD and YPO grown cells contain the same sets of proteins? (ii) Are there specific LP proteins present only in LP from YPD or YPO grown cells, respectively? If this was the case, can we learn something about the change in LP associated metabolism caused by the change of the substrate from (possible) functions of these specific LP proteins? (iii) Finally, does this strategy allow us to identify novel LP proteins.

Answers to the questions mentioned above can only be given in part. Uncertainties are due to the fact that despite the sophisticated method of proteome investigation employed in this study some polypeptides may have escaped detection. Moreover, slight contamination of LP with other organelles may still lead to false results. Although all samples used for protein mass spectrometry were rigorously tested for cross-contamination we are aware of the fact

that 100 % purity can never be warranted. Despite these caveats, data summarized in Table 6 provide some valuable and novel information. In LP from YPD grown cells 49 polypeptides were identified, and in LP from YPO grown cells 54 proteins. As can be clearly seen from Table 6 and Fig. 4, only 26 of these proteins occurred in both LP variants. Interestingly, most of these proteins are involved in lipid metabolism, such as Erg-proteins, lipases or fatty acid activating proteins. Six proteins detected in both types of LP have not been attributed to LP before, namely Cpr5p, Gtt1p, Osh4p, Ubx2p, Vps66p, Ypt7p. Two of these proteins, Osh4p and Vps66, were previously found to be localized to the LP of *Yarrowia lipolytica* (Athenstaedt *et al.*, 2006). Preliminary results employing GFP-fluorescence microscopy showed that in *Saccharomyces cerevisiae* Gtt1p, Osh4p, Vps66p and Ypt7p formed distinct punctuate structures when grown to stationary phase on glucose containing media. Whether these structures overlap indeed with LP needs to be verified with dye merging and cell fractionation experiments.

The question as to the occurrence of specific groups of proteins in LP from YPD or YPO grown cells, respectiviely, remained. The major reason for this uncertainty is the insufficient annotation of many of these orphan proteins. Gene ontology annotations of the newly found putative LP proteins revealed that they covered a broad spectrum of biological processes under both growth conditions. One big group is associated with lipid metabolic processes. Surprisingly, a shift towards oleic acid brought about a decrease in proteins of this kind. On the other hand, a remarkable increase in proteins involved in processes of translation was found in LP from YPO grown cells. It has also to be noted that hardly any protein is known that is explicitly localized to LP. Erg6p for example, a very prominent LP protein, is known to localize to ER (McCammon *et al.*, 1984) and mitochondria (Sickmann *et al.*, 2003) besides the LP (Athenstaedt *et al.*, 1999). Another example is the long chain fatty acyl-CoA synthetase Faa1p which localized to the LP (Athenstaedt *et al.*, 1999), mitochondrion

(Sickmann *et al.*, 2003) and the plasma membrane (Delom *et al.*, 2006). This characteristic propertiy of LP proteins complicates a direct assignment of putative new proteins to this cellular compartment, and data have therefore to be interpreted with caution.

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Tables

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source
BY4741	*Mata lys2*Δ0 *leu2* Δ0 *ura3* Δ0 *his3*Δ1	EUROSCARF
ATCC 201388 (OSH4-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *OSH4-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (VPS66-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *VPS66-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (CPR5-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *CPR5-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (GTT1-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *GTT1-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (UBX2-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *UBX2-GFP::HIS3MX*	INVITROGEN
ATCC 201388 (YPT7-GFP)	*Mata his3*Δ1 *leu2* Δ0 *met15Δ0 ura3*Δ0 *YPT7-GFP::HIS3MX*	INVITROGEN

Table 2. Lipid composition of homogenate and lipid particles of *Saccharomyces cerevisiae* wild type grown on either YPD or YPO

mg / mg protein		
	Glucose	Oleate
Triacylglycerols	32.0 +/- 4.0	97.3+/- 8.9
Steryl esters	36.7 +/- 4.1	1.0 +/- 0.3
mg phospholipid / mg protein		
	Glucose	Oleate
Homogenate	0.047 +/- 0.003	0.071 +/- 0.004
Lipid particle	0.423 +/- 0.048	0.889 +/- 0.054

Table 3. Phospholipid composition of homogenate and LP isolated from cells grown on glucose (YPD) or oleate (YPO)

	% of total phospholipids			
	Homogenate		Lipid particle	
	YPD	YPO	YPD	YPO
PA	2.8 +/- 0.4	0.7 +/- 0.7	1.8 +/- 1.3	1.3 +/- 2.7
PI	14.5 +/- 5.9	16.9 +/-3.8	21.5 +/- 3.4	21.5 +/- 3.4
PS	3.8 +/- 0.4	3.3 +/- 0.9	2.1+/- 2.6	0.8 +/- 0.9
PC	51.1 +/- 5.5	53.0 +/- 1.4	57.5 +/- 1.7	56.4 +/- 2.7
PE	23.6 +/- 1.4	20.1 +/- 3.7	16.6 +/- 1.9	16.9 +/- 2.8
CL	2.3 +/- 0.3	3.7 +/- 0.8	0 +/- 0	1.0 +/- 1.2
LP	0 +/- 0	0.3 +/- 0.6	0.3 +/- 0.6	0.7 +/- 0.5
DMPE	1.9 +/- 1.2	1.5 +/- 1.4	0 +/- 0	1.3 +/- 1.9

Table 4. Degree of saturation of phospholipids from homogenate and LP isolated from cells grown on glucose or oleic acid

	% of saturated fatty acids			
	Homogenate		Lipid particle	
	Glucose	Oleate	Glucose	Oleate
PE	17.5	5.4	7.6	2.3
PC	17.2	5.6	10.6	3.0
PI	42.4	26.5	40.4	33.8
PS	35.9	20.7	34.9	10.8

Table 5. Proteome of yeast LP. [§]novel LP protein, found on glucose as well on oleate grown cells, [¥] numbers indicate fragments used for identification of proteins from cells grown on glucose (D) or oleate (O). C..cytosol;M..mitochondria; PM..plasma membrane; ER..endoplasmic reticulum; LP..lipid particle; End..endosomes; G..golgi; Mic..microsomes; V..vacuole; Px..peroxisome; N..nucleus; nEnv..nuclear envelope; ext..extrinsic to membrane; mem..integral to membrane; bud..cellular bud; rib..ribosomal subunit; CW..cell wall; R..ribosome; Retro..retrotransposon; mTub..microtubule

Gene name	Systematic name	SGD GFP YPL MIPs	This study	D[¥]	O[¥]	Localisation (SGD)	Description
ACH1	YBL015W		✓		2	C / M	CoA transferase activity
ADH1	YOL086C		✓		2	C / PM	Alcohol dehydrogenase
ALG9	YNL219C		✓	2		ER	Mannosyltransferase
ATF1	YOR377W	✓				LP / End	Alcohol acyltransferase
ATP2	YJR121W				9	M	Subunit of mitochondria ATP synthase
AYR1	YIL124W	✓ ✓	✓	15	12	C / ER / LP / M	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase
BSC2	YDR275W	✓ ✓ ✓				LP	Unknown
COY1	YKL179C	✓ ✓				G	Golgi membrane protein
CPR5[§]	YDR304C		✓	7	3	C / ER	Peptidyl-prolyl cis-trans isomerase
CSR1	YLR380W	✓				LP / C / M / Mic	Phosphatidylinositol transfer protein
CST26	YBR042C	✓ ✓ ✓ ✓				LP	Required for incorporation of stearic acid into phosphatidylinostiol
CWH43	YCR017C		✓	2		PM	Putative sensor/transporter protein
DFM1	YDR411C		✓	2		ER	ER localized derlin-like family member
DGA1	YOR245C	✓	✓		3	LP	Diacylglycerol acyltransferase
DPL1	YDR294C		✓	2		ER	Dihydrosphingosine phosphate lyase
DPM1	YPR183W		✓	2		ER / M	Dolichol phosphate mannose synthase
EHT1	YBR177C	✓ ✓ ✓	✓	19	18	LP / M	Acyl-coenzymeA:ethanol O-acyltransferase

ENO2	YHR174W					✓		2	V / PM / M	Enolase II
ERG1	YGR175C	✓		✓	✓	✓	5	15	ER / LP	Squalene epoxidase
ERG6	YML008C	✓	✓	✓	✓	✓	37	30	ER / LP / M	Delta(24)-sterol C-methyltransferase
ERG7	YHR072W	✓	✓	✓	✓	✓	7	2	ER /LP / PM	Lanosterol synthase
ERG27	YLR100W		✓	✓	✓	✓	9	4	ER / M	3-Keto sterol reductase
FAA1	YOR317W	✓				✓	20	15	LP / PM / M	Long chain fatty acyl-CoA synthetase
FAA3	YIL009W					✓	3		unknown	Long chain fatty acyl-CoA synthetase
FAA4	YMR246W	✓	✓	✓	✓	✓	13	6	LP / C	Long chain fatty acyl-CoA synthetase
FAT1	YBR041W	✓	✓	✓	✓	✓	13	9	PM / LP / Mic / PX	Fatty acid transporter
FMP52	YER004W					✓		4	ER / M	Unknown
GPT2	YKR067W					✓	12		C / ER	sn-1 Acyltransferase
GTT1[§]	YIR038C					✓	2	8	ER / M / PM	glutathione S-transferase
GVP36	YIL041W					✓		3	C / G	BAR domain-containing protein
HFD1	YMR110C	✓	✓		✓	✓	7	18	M / LP / End	Putative fatty aldehyde dehydrogenase
HSP12	YFL014W					✓		6	C / PM / N	Heat shock protein
KAR2	YJL034W					✓	9		ER	ATPase
LAP4	YKL103C					✓		3	V	Vacuolar aminopeptidase
LDB16	YCL005W	✓	✓		✓				LP / M	Unknown
MSC1	YML128C					✓	2		M / ER / PM	Unknown
NUS1	YDL193W	✓		✓		✓	9	4	ER / LP /nEnv	Putative prenyltransferase
OSH4[§]	YPL145C						3	5	C / G / ext	Oxysterol binding protein
OSW5	YMR148W		✓		✓	✓	2		mem	Unknown
PDI1	YCL043C					✓	6	7	ER	Disulfide isomerase
PDR16	YNL231C	✓	✓		✓	✓	5	9	LP / Mic / PM / C	Phosphatidylinositol transfer protein
PET10	YKR046C	✓	✓	✓	✓	✓	12	24	LP	Unknown

PGC1	YPL206C	✓	✓	9		LP / M	Phosphatidylglycerol phospholipase C
PIL1	YGR086C	✓				C / M / PM	Primary component of eisosomes
PMA1	YGL008C		✓		3	PM / M / mem	Plasma membrane H+-ATPase
PMT1	YDL095W		✓	2		ER	Protein O-mannosyltransferase
PMT2	YAL023C		✓	3		ER	Protein O-mannosyltransferase
POR1	YNL055C		✓		2	M	Mitochondrial porin
POX1	YGL205W		✓		3	PX	Fatty-acyl coenzyme A oxidase
RHO1	YPR165W		✓	4		mem / PX / PM / M / bud	GTP-binding protein
RPL5	YPL131W		✓		3	rib	Protein component of the large (60S) ribosomal subunit
RPL10	YLR075W		✓		2	rib	Protein component of the large (60S) ribosomal subunit
RPS1B	YML063W		✓		2	rib	Ribosomal protein 10 (rp10) of the small (40S) subunit
RPS3	YNL178W		✓		5	rib	Protein component of the small (40S) ribosomal subunit
RPS19B	YNL302C		✓	2		rib	Protein component of the small (40S) ribosomal subunit
RPS31	YLR167W		✓		4	rib / C	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin
RRT8	YOL048C	✓ ✓ ✓	✓	4	2	LP	Unknown
RTN2	YDL204W		✓	3		ER / nEnv	Unknown
SEC61	YLR378C		✓	5		ER	Essential subunit of Sec61 complex
SEC63	YOR254C		✓	4		ER / M	Essential subunit of Sec63 complex
SHE10	YGL228W		✓	4		unknown	Putative glycosylphosphatidylinositol (GPI)-anchored protein of unknown function

SLC1	YDL052C	✓		✓	✓	✓	2	3	LP	1-Acyl-sn-glycerol-3-phosphate acyltransferase
SNA2	YDR525W-a	✓	✓		✓				Mem / C	Unknown
SNX41	YDR425W	✓			✓				End	Sorting nexin
SRT1	YMR101C	✓							LP	Cis-prenyltransferase
SSA1	YAL005C					✓		2	C / PM / N	ATPase
SSO1	YPL232W		✓		✓				PM	Plasma membrane t-snare
TDH1	YJL052W	✓				✓		4	C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 1
TDH2	YJR009C	✓							C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2
TDH3	YGR192C	✓				✓		5	C / LP / M / PM / CW	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3
TEF1	YPR080W							5	M / R	Translational elongation factor EF-1 alpha
TGL1	YKL140W	✓			✓	✓	12	14	LP / mem	Steryl ester hydrolase
TGL3	YMR313C	✓	✓		✓	✓	4	5	LP	Triacylglycerol lipase
TGL4	YKR089C	✓	✓		✓	✓	3	4	LP	Triacylglycerol lipase
TGL5	YOR081C	✓	✓		✓	✓	5	3	LP	Triacylglycerol lipase
USE1	YGL098W		✓		✓				ER	SNARE protein
TSC10	YBR265W					✓	2		C / ER / M	3-ketosphinganine reductase
TUB2	YFL037W					✓		2	mTub	Beta-tubulin
UBX2[§]	YML013W					✓	5	3	ER / M	Protein involved in ER-associated protein degradation
VPS66[§]	YPR139C					✓	6	4	C	Cytoplasmic protein of unknown function involved in vacuolar protein sorting
WBP1	YEL002C					✓	3		ER / nEnv	Beta subunit of the oligosaccharyl transferase (OST) glycoprotein complex

YBR204C	YBR204C		✓		3	unknown	Serine hydrolase
YDR018C	YDR018C	✓				unknown	Similarity to acyltransferase
YEH1	YLL012W	✓ ✓ ✓				LP / mem	Steryl ester hydrolase
YIM1	YMR152W	✓ ✓	✓		4	LP / C / M	Unknown
YGR038C-B	YGR038C-B		✓	2		Retro	Retrotransposon TYA Gag and TYB Pol genes
YJU3	YKL094W	✓ ✓ ✓	✓	3	2	LP / C / M / PM	Serine hydrolase
YNL134C	YNL134C		✓		2	C / N	Unknown
YNL208W	YNL208W		✓		3	M / R	Unknown
YOR059C	YOR059C	✓				LP	Unknown
YOR246C	YOR246C	✓ ✓ ✓				LP	Similarity to oxidoreductase
YPT7[§]	YML001W		✓	4	2	V / M	GTPase
ZEO1	YOL109W		✓		9	M / PM / ext	Peripheral membrane protein

Figures

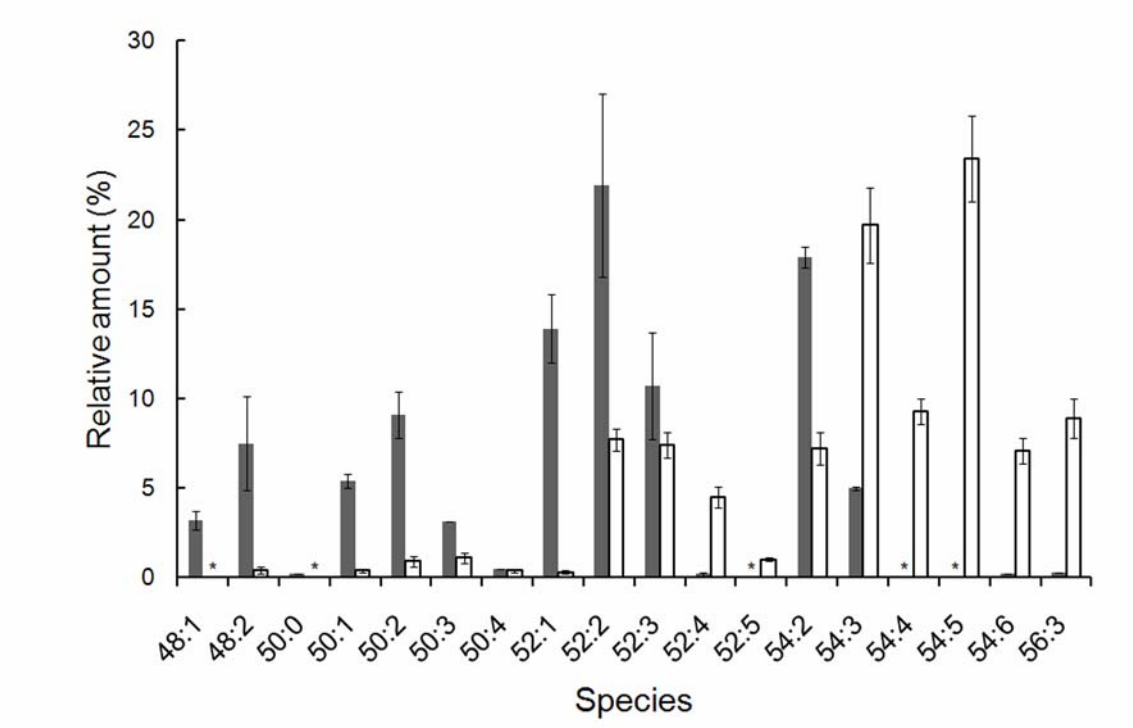

Figure 1

Oleic acid has great impact on the formation of TAG species.

TAG of LP from wild type cells grown on glucose (dark bar) or oleate (white bar) were analysed by MS. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

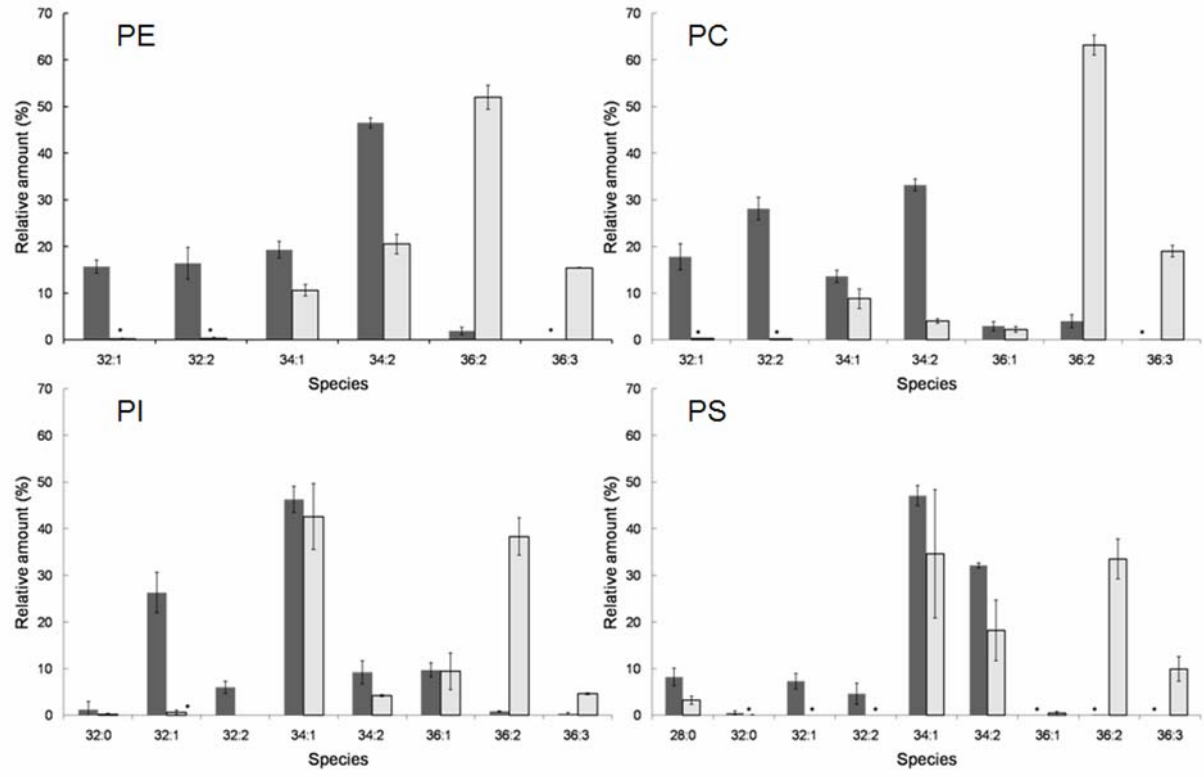

Figure 2

Molecular composition of phospholipid species from homogenates.

Lipid extracts of homogenate from wild type cells grown on either glucose (grey bar) or oleate (white bar) were analysed by MS for phospholipid (PE, PC, PI, PS) species. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

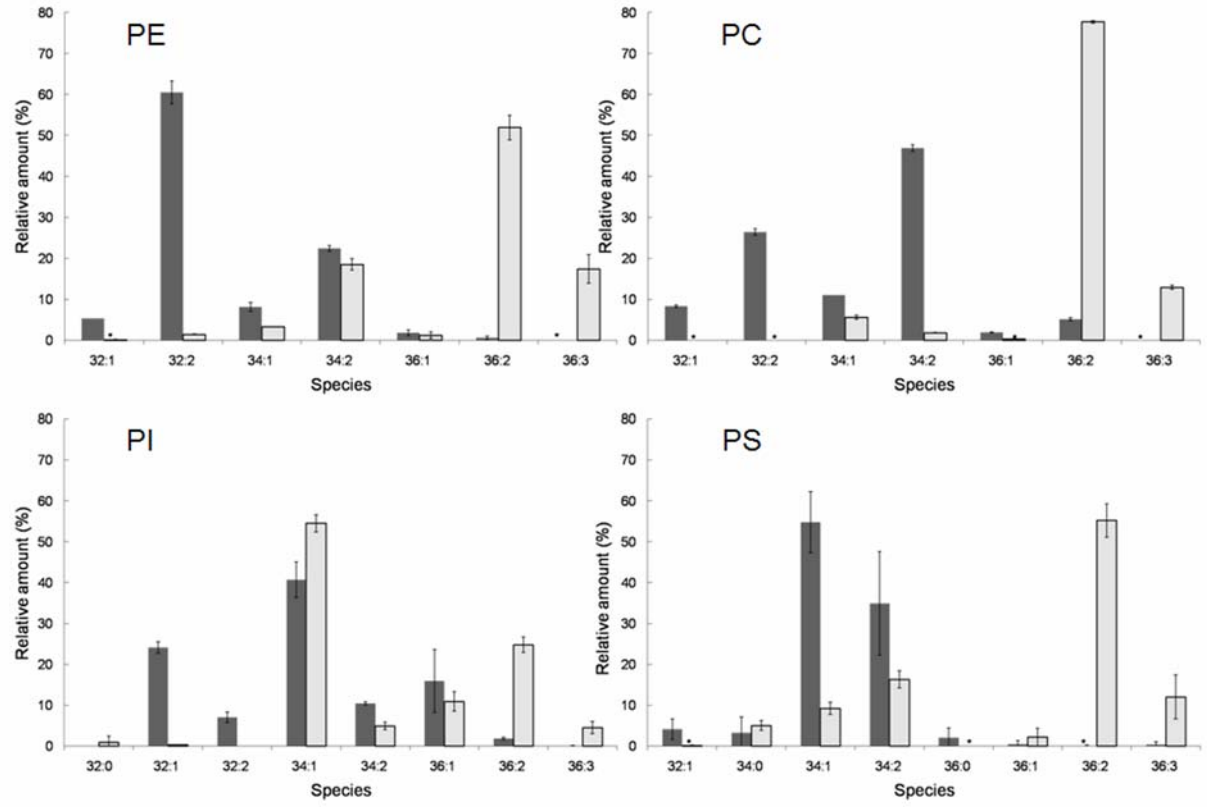

Figure 3

Molecular composition of phospholipid species from lipid particles.

Lipid extracts of LP from wild type cells grown on either glucose (dark bar) or oleate (grey bar) were analysed by MS for phospholipid (PE, PC, PI, PS) species. Data are mean values from at least 2 independent experiments. *= values are ≤ 0.1%

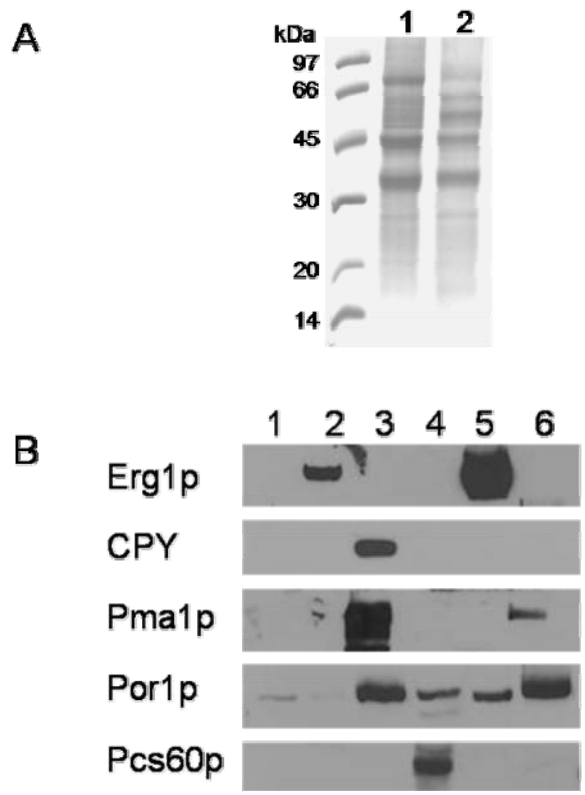

Figure 4

Protein analysis and quality control.

A, Protein patterns of lipid particle fraction from *Saccharomyces cerevisiae* wild type cells grown on glucose (1) or oleic acid (2). Low molecular weight standard was loaded in the first lane. Lanes were loaded with 15 µg total protein, each. B, Quality control of subcellular fractions from *Saccharomyces cerevisiae* wild type cells. Western blot analysis of cell fractions from BY4741 grown on glucose (1-3) and oleic acid (4-6). Homogenate (1, 4), lipid particle (2,5) and vacuole (3,6). Lanes were loaded with 15 µg total protein, each.

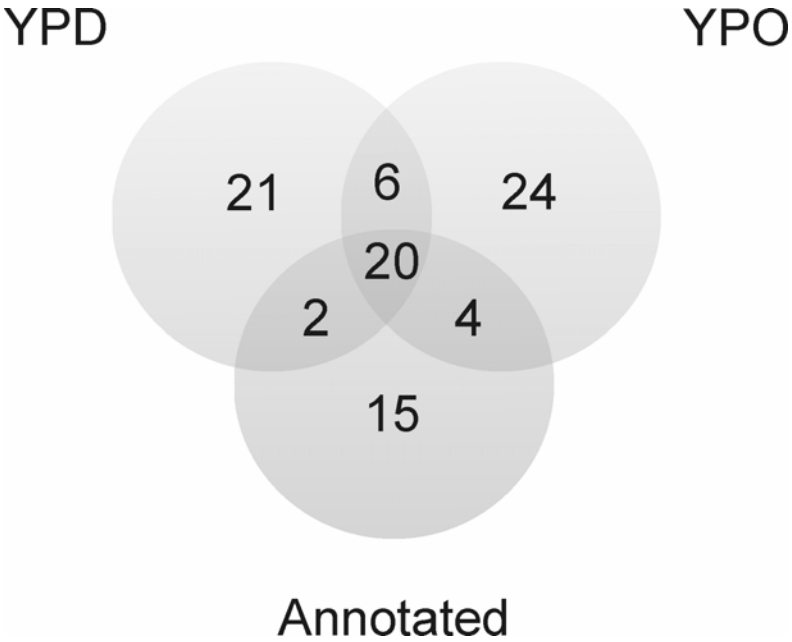

Figure 5

Venn diagram displaying results of MS-analysis of LP.

The graph summarizes LP protein analysis from cells grown on glucose (YPD) and oleate (YPO).

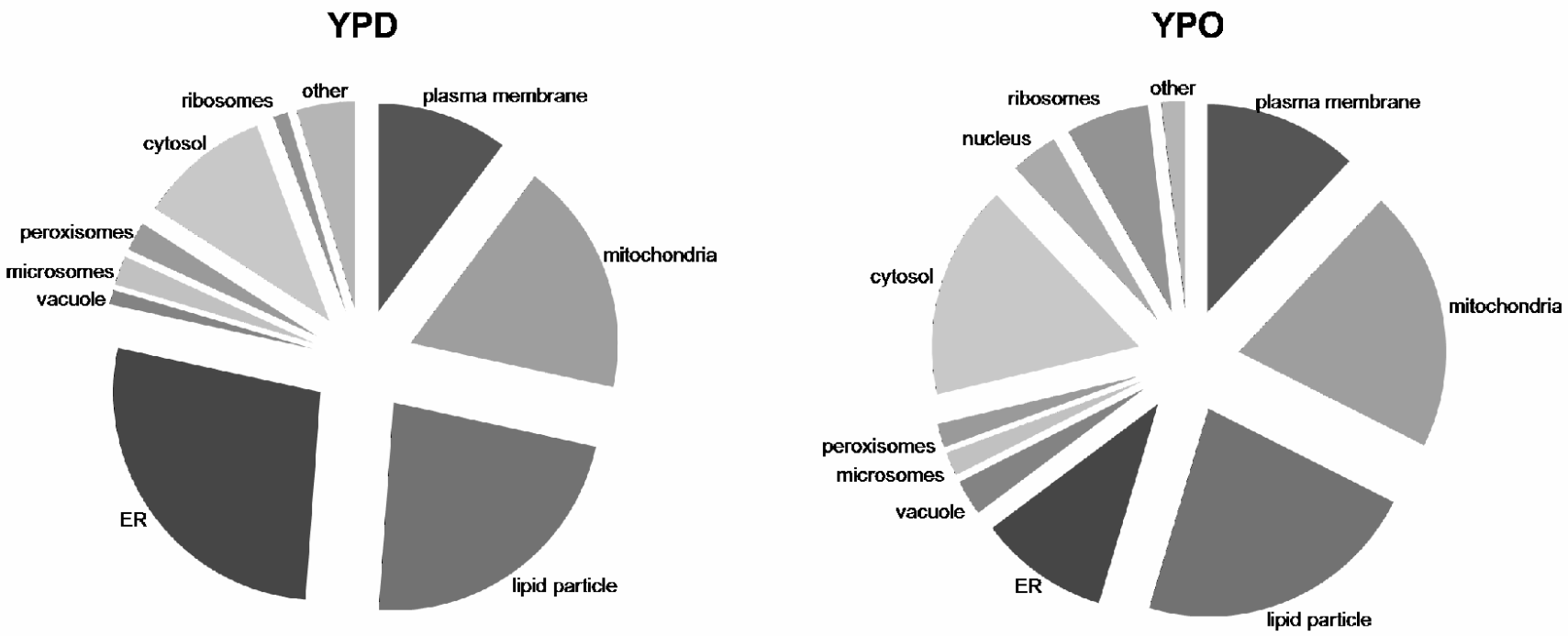

Figure 6

Assignment to subcellular fractions of LP proteins from wild type cells grown on glucose (YPD) or oleate (YPO).

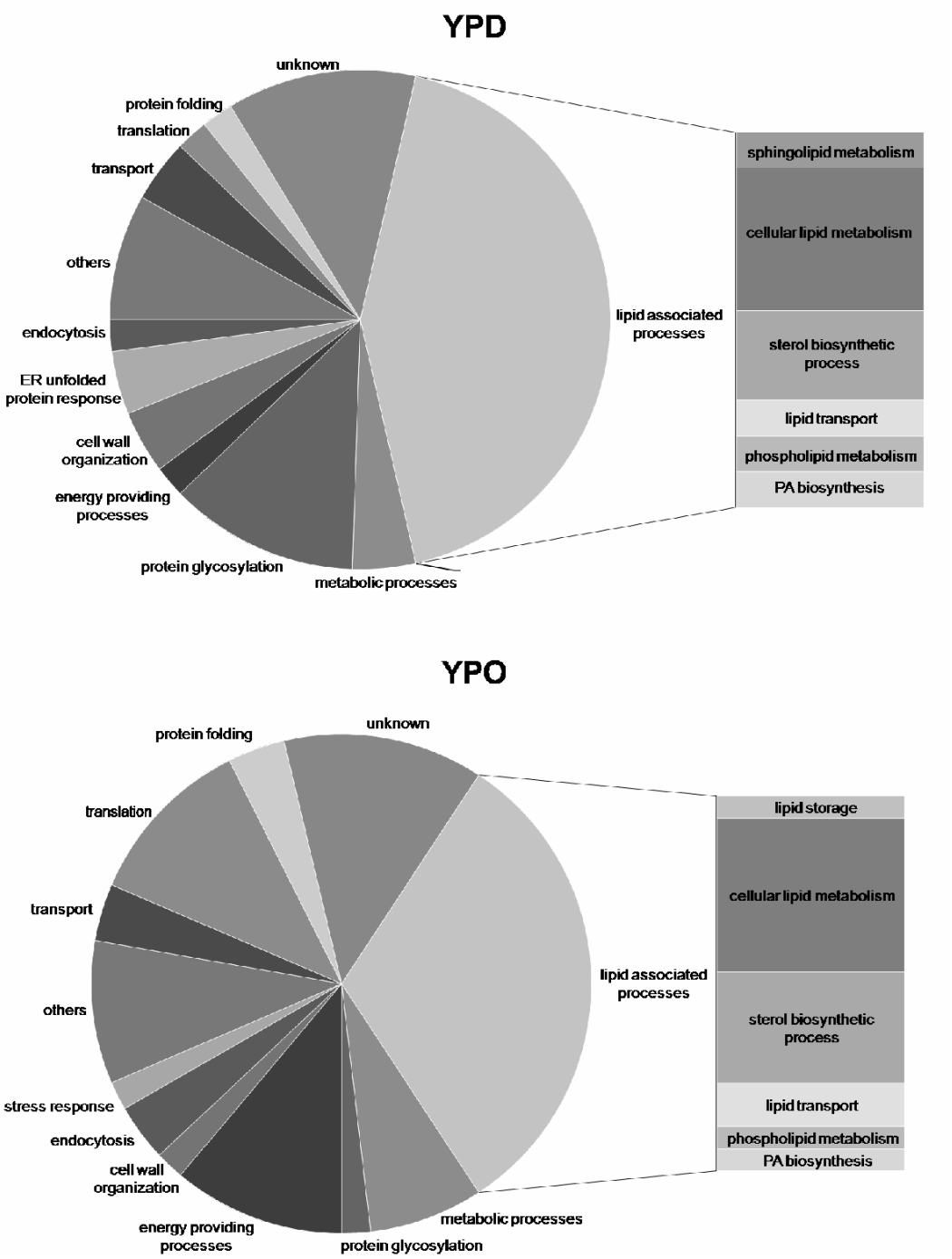

Figure 7

Assignment of newly found proteins of LP from wild type cells grown on glucose (YPD) or oleate (YPO) to biological processes.

Chapter 6

Membrane topology study of yeast lipid particle protein Erg1p

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Key words: lipid particle, yeast, hydrophobic, protein, topology

Abbreviations: LP: lipid particles; ER: endoplasmic reticulum; aa: amino acid

Abstract

In yeast as in most other eukaryotic cells neutral lipids triacylglycerols (TAG) and steryl esters (STE) are stored in so-called lipid particles (LP). In contrary to other subcellular organelles LP are not surrounded by a phospholipid bilayer, but by a monolayer. To improve our overall picture of the structural organization of lipid particles we started to investigate the surface membrane proteins of this compartment in more detail. These proteins are embedded within the monolayer membrane of lipid particles, but they neither contain targeting signals nor membrane anchors which direct them to their proper destination. As a model enzyme for these topology studies we used Erg1p, squalene epoxidase, which is a typical yeast lipid particle protein. Preliminary results give some hints on the orientation of terminal parts of the protein. They show that large portions of the protein are exposed to the surface of the LP monolayer membrane, and only a smaller part is protected by the membrane. These studies will help to elucidate biogenesis and function of yeast lipid particles in general as well as the specific enzymology of Erg1p.

Introduction

Proteins are known to be attached or integrated within biological membranes by various mechanisms. The textbook suggests a general classification of membrane proteins as integral membrane proteins (also known as intrinsic proteins) and peripheral proteins (or extrinsic proteins). Integral membrane proteins contain one or more transmembrane domains, spanning the entire phospholipid bilayer. Characteristics of these membrane spanning domains are hydrophobic stretches which interact with the fatty acyl groups of the membrane phospholipids and anchor the protein to the membrane. All transmembrane proteins that have been investigated so far contain either $\alpha-$helices or multiple β-strands within their membrane spanning domains. Exceptionally, some intrinsic proteins are not spanning the phospholipid bilayer, but are covalently bound to fatty acids which are embedded in the membrane. Instead of interacting with hydrophobic parts of the phospholipid bilayer, peripheral membrane proteins are indirectly bound to integral membrane proteins or lipid polar head groups [http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mcb&part=A608]. Other processes which are important for associating proteins to membranes are covalent attachments of lipid moieties. Four major forms of lipid modification have currently been described in eukaryotic cells: (N)-terminal myristoylation of cytosolic proteins occurs co-translationally; plasma membrane proteins can be modified with glycosylphosphatidylinositol (GPI); cytoplasmic proteins can be isoprenylated at their (C)-terminus; and palmitic acid can be added in an post-translational event to many integral and peripheral membrane proteins (Bhatnagar and Gordon, 1997; Resh, 1999). The mechanisms listed above describe how proteins can be embedded into phospholipid bilayers. However, it remains uncertain how proteins can be anchored to phospholipid monolayers.

Yeast as well as higher eukaryotic cells (mammals and plants) contain an organelle that is protected by a phospholipid monolayer, the lipid particle (LP), lipid droplet or oil body. The yeast lipid particle functions as a storage compartment for neutral lipids, triacylglycerols (TAG) and steryl esters (STE) forming the hydrophobic core of this organelle, but is also actively involved in metabolic processes of lipid homeostasis. A small but specific set of approximately 40 proteins is so far annotated as localized at least partially to the yeast LP. Recent data, however, suggest that this list has to be extended (Grillitsch *et al*., manuscript in preparation).

LP proteins from yeast do not possess a common signalling motif unlike their counterparts in mammals or plants nor do they contain transmembrane regions which could target them to their final destination. Proteins of mammalian lipid droplets have typical representatives, such as proteins of the PAT family (perilipin, adipophilin, and the tail-interacting protein of 47 kDa). Typical plant LP proteins are the oleosins. For distinct targeting to the LP in both cases well defined hydrophobic domains are required, that are either sufficient by themselves (Subramanian *et al.*, 2004; Zehmer *et al.*, 2008), require the presence of a proline knot motif (PKM), e.g. with oleosins (Abell *et al.*, 1997; Abell *et al.*, 2004), or contain polar sequences which flank the hydrophobic stretches (Ingelmo-Torres *et al.*, 2009). Müllner *et al.* (Müllner *et al.*, 2004) showed that certain hydrophobic stretches near the C-terminus of three major yeast LP proteins (Erg1p, Erg6p and Erg7p) are important for proper localization to LP. When these proteins were truncated for their hydrophobic parts they were retained to the endoplasmic reticulum (ER). Besides the importance of hydrophobic domains no other common structural features have so far been identified for yeast LP proteins.

One of the most prominent proteins, that can be found in yeast LP and the ER, is Erg1p (squalene epoxidase) (Ruckenstuhl *et al.*, 2007). Topology studies (described here)

using Erg1p as a model for LP proteins revealed that most parts of Erg1p are facing the cytosol. Understanding the protein orientation may help in structure-function studies of LP associated proteins.

Materials and Methods

Yeast strains and culture conditions

Yeast strains used throughout this study are listed in Table 1. Cells were grown at 30°C in rich medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD) or in minimal glucose medium containing 0.67% yeast nitrogen base (Difco), 2% glucose and the appropriate mixture of amino acids. To induce overexpression of *ERG1* the carbon source in minimal medium was changed to 2% galactose.

Plasmid construction

Plasmids (see Table 2) were constructed using standard molecular biological procedures. For generating plasmids containing *ERG1* constructs with internal HA-tags, *ERG1* was produced from two PCR fragments, fragment 1 and fragment 2. The sequence encoding YPYDVPDYA (HA, haemagluttinin) was part of the amplification oligonucleotides to generate two PCR products overlapping in the sequence of the tag (see Table 3). After fusion of fragment 1 and 2 in an overlapping PCR step, the final fragment was cloned into expression vector pESC-LEU, as the oligonucleotides of the last PCR step contained restriction sites corresponding to the multiple cloning site (5´-end of PCR fragment carried *Bam*HI site and 3´-end carried *Sal*I site). All plasmid constructs were confirmed by DNA sequencing.

Site-directed mutagenesis

The inactivation of the native fXa recognition site (IDGR at aa 192-195) was achieved by substitution of Ile-192 by Val and substitution of Gly-194 by Ala. Substitutions

were generated by QuickChange mutagenesis (Stratagene). Mutants were confirmed by DNA sequencing.

Subcellular fractionation of yeast cells

Subcellular fractions of yeast cells were prepared by published procedures (Leber *et al.*, 1994; Zinser and Daum, 1995) and routinely tested by Western blot analysis (Haid and Suissa, 1983) using rabbit antibodies against Erg1p, Por1p, Prc1p/CPY and Wbp1p. Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection reagents (SuperSignal^{TM}, Pierce Chemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

Protein analysis

Proteins from isolated subcellular organelles were precipitated with trichloroacetic acid at a final concentration of 10%. Proteins were quantified by the method of Lowry *k.* (LOWRY *et al.*, 1951) using bovine serum albumin (BSA) as standard. Prior to protein analysis, samples of LP were delipidated. Non-polar lipids were extracted with two volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were analyzed as described above. SDS-PAGE was carried out as described by Laemmli *et al*. (Laemmli, 1970) using 12.5% separation gels. After SDS-PAGE, proteins samples were transferred to nitrocellulose transfer membranes (Pall). The membranes were than subjected to immunoblot analysis, and antisera were used at the following dilutions: anti-Erg1p (1:30.000), anti-TAP (1:5.000), anti-GFP (1:500) and anti-HA11 (1:5.000). Immunoblots were washed and incubated with secondary anti-rabbit or anti-mouse Ig horseradish peroxidase linked antibody and developed using detection reagents as described above.

Proteolytic treatment with proteinase K and factor Xa protease

Aliquots of LP fractions (10 µg protein) were incubated with proteinase K (0.3 mg/ml, Macherey Nagel) in the presence or absence of Triton X-100 (0.4%) for 5 min at 4°C. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 50 mM. Proteins were analyzed after delipidation as described above. For the fXa treatment 100 µg protein of 40,000 g microsomal fractions were diluted at a ratio of 1:3 with fXa-buffer (250 mM sorbitol, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA), or 10 µg protein of LP fractions were used. Samples were mock-digested or digested with 1 µg of factor Xa protease (NewEngland biolabs) in the presence or absence of 0.2% Nonidet P-40 (NP-40) at room temperature for 3 hours. The reaction was stopped with PMSF and further analyzed as described above.

Determination of the glycosylation status of native Erg1p

100 µg protein from 30,000 g and 40,000 g microsomal preparations were adjusted with 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA to a final volume of 54 µl. Six µl of 10x denaturing buffer (5% SDS, 10% beta-mercaptoethanol) were added, and the samples were incubated at 95°C for 10 min. 10µl of 10x G5 buffer (0.5 M sodium citrate, pH 5.5) were added to the boiled samples, and the samples were split into 2 aliquots. One aliquot was treated with 2 µl of Endo H (1 U/200 µl, Roche), and the other aliquot was incubated with buffer. Samples were incubated at 37°C for 2 hours, and proteins were separated by SDS-PAGE gel electrophoresis and visualized by immuno-blotting using antibodies against Erg1p and Wbp1p.

Immunofluorescence

Yeast cells were grown overnight on leucine-free minimal medium containing 2% glucose. For induction of Erg1p variants carrying internal HA-tags, cells were harvested and inoculated into leucine-free minimal medium containing 2% galactose. Cells were grown to the stationary phase, formaldehyde was added to a final concentration of 3.7%, and incubation was continued for 30 min. Fixed cells were harvested, washed and resuspended in spheroplasting buffer (1.2 M sorbitol, 100 mM KPi) and incubated with zymolyase (0.1 mg/ml, Seikagaku Corp., Tokyo, Japan) for 30 min at 30°C. Spheroplasts were washed twice with phosphate-buffered saline (PBS), blocked with PBS/1% BSA and placed on polyethylenimine-coated multiwell slides. Immunolabeling was carried out by using monoclonal antibody against HA (Covance, HA11, diluted 1:100 in PBS/1% BSA) overnight at 4°C. Cells were washed with PBS and 1% BSA and incubated with the secondary antibody, Goat anti-mouse IgG, DyLight^{TM}488 (Pierce, dilution 1:100), in PBS/1% BSA for 1 hour at room temperature. After washing once with PBS/1% BSA and twice with PBS, cells were mounted in 80% glycerol. Fluorescence microscopy was performed on a Zeiss Axiovert 35 microscope using a 100-fold oil immersion objective, a UV lamp, and a decetion range between 450 nm and 490 nm. Images were taken with a CCD camera.

Results

Membrane topology prediction for Erg1p

Erg1p is a squalene-monooxygenase that localizes dually, namely to the endoplasmic reticulum (ER) and the lipid particle (LP), and is restricted in its activity to the former compartment (Leber *et al.*, 1998). It is essential for the synthesis of ergosterol in fungi similar

to its mammalian orthologs where it is inevitable for the production of cholesterol. Squalene epoxidase catalyzes the conversion of squalene to 2,3-oxidosqualene in an oxygen, FAD and either NADH or NADPH (organism dependent) required manner (Ruckenstuhl *et al.*, 2005). Determination of the topology of Erg1p in different organelles is an important step to explain enzyme activity in different locations. A first hint how Erg1p may be embedded within the membrane, at least in the bilayer of the ER membrane, was prediction of very hydrophobic areas using the Kyte-Doolittle-Hydropathy Plot (Figure 1). At both termini hydrophobic stretches are present which resemble transmembrane domains. When Erg1p was truncated for its C-terminal hydrophobic domains the protein was retained to the ER (Müllner *et al.*, 2004), but ER residence was not affected at all.

Proteolysis of LP proteins

Unlike other subcellular organelles, LP are not covered by a phospholipid bilayer but by a monolayer that protects the hydrophobic core of non-polar lipids from the surrounding (Czabany *et al.*, 2008). The high fat content of LP which constitutes more than 95 % of the total mass hinders easy handling in topology studies (Leber *et al.*, 1994; Zweytick *et al.*, 2000). In initial experiments, LP were incubated with proteinase K (see Methods and Materials), proteolysis was stopped by addition of excessive amounts of PMSF and proteins analysed by SDS-PAGE after delipidation. Figure 2 shows the protein pattern of LP that were either treated with proteinase K (+) or mock-digested (-). In this experiment, the incubation time was raised to 3 hours at room temperature. As the patterns differed greatly it was obvious that the effect of the protease on single LP proteins could not be generalized. Some proteins seemed to be more protected against the protease than the majority of the other LP proteins.

Orientation of Erg1p on LP

Further analysis regarding topology of LP proteins work was restricted to Erg1p, one of the most prominent yeast LP proteins. Additional proteolysis experiments with proteinase K were conducted to investigate the localization of the termini of the protein on LP. LP of strains expressing Erg1p variants that were N- or C-terminally tagged were subjected to limited proteolysis. Susceptibility of tags to the proteinase was visualized by immuno-detection after proteolytic cleavage. Figure 3 summarizes these experiments. Proteinase K had access to the C-terminal TAP (Tandem Affinity Purification) tag independent of the presence of solubilising detergent Triton X-100 (Figure 3A). In order to reduce possible negative effects of the tag used for designing the constructs the same experiment was performed using a different tag. Same results were obtained when the TAP-tag used for this experiment was replaced by a GFP-tag (Figure 3B). Incubating LP that carried N-terminally GFP-tagged Erg1p with proteinase K brought about the same result. Sensitivity to the proteinase K was shown in the presence or absence of detergent as no protected immunoreactive protein fragments were detected in either case (Figure 3C). This result indicated that both termini of Erg1p are oriented towards the cytosol.

Analysis of internally tagged Erg1p-HA fusion proteins

An additional approach for expanding the information on the topology of Erg1p that localizes to the LP was investigation of fusion proteins that contained a very short tag (HA, haemagluttinin) within its internal sequence. HA tags were inserted in-frame at nucleotide positions 414 and 1140 that were both next to hydrophobic and hence putative membrane-spanning regions (see Materials and Methods). The respective Erg1p constructs were overexpressed with a high copy vector system in the presence of native but untagged Erg1p upon galactose induction. LP were isolated as described in Methods section and subjected to

proteinase K treatment. Both internally tagged Erg1p variants clearly showed high sensitivity to protease treatment. In both cases total digestion of the tags occurred independent of the presence of detergent (see Figure 4A and 4C). These results indicated that both positions marked by HA tags were easily accessible for the protease and most likely oriented towards the cytosol. For additional verification of these results immunofluorescence experiments were conducted. Access of the antibody to the HA-tag indicates outside orientation, whereas inside orientation would result in no signal. In Figures 4B and 4D proper binding of the antibody to the HA-tag could be seen for both Erg1p variants, thus confirming findings of the proteolysis experiment.

Analysis of Erg1p topology by native fXa site

Erg1p contains at amino acid (aa) 64 a single cleavage site (IE/DGR) for the specific protease fXa. Cleavage of Erg1p with this protease was tested with antisera against Erg1p and could be performed without previous tagging of the protein. LP fractions and 40,000 g microsomes of wild type yeast BY4741 were treated with protease fXa in the absence or presence of detergent. Subsequent Western Blot analysis showed reduced levels of Erg1p when treated with protease fXa irrespective of the sample´s origin (see Figure 5). Total cleavage of Erg1p was only achieved in samples derived from ER which were treated with detergent, whereas LP derived Erg1p was not totally cleaved but strongly reduced. As levels of Erg1p were not unaltered when treated with protease fXa only results remained unclear. The recognition site at aa-position 64 appears to be at least partially protected and not completely accessible to protease fXa.

Glycosylation status of ER resident Erg1p

A common method for analyzing the topology of ER resided proteins is the appliance of glycosylation tags. Eukaryotic cells show glycosylation activity that is restricted to the lumen of the ER and performed by oligosaccharyl transferase (OST). This enzyme adds oligosaccharides to asparagine residues of the consensus sequence Asn-X-Thr/Ser (Welply *et al.*, 1983; van Geest and Lolkema, 2000), at least when they are oriented towards the lumen of the ER. Analyzing the protein pattern after treatment with enzymes that cleave glycosylations helps to distinguish between glycosylated and non-glycosylated forms. Native Erg1p contains two potential glycosylation sites, one at aa-position 159 (-Asn-IleThr-) and the other at aa-position 166 (-Asn-Val-Thr-). 30,000 g and 40,000 g microsomal fractions of wild type yeast were treated with Endoglycosidase H as described in Materials and Methods. Erg1p of the fractions analyzed did not show an altered gel mobility after treatment with endo H (see Figure 6). The positive control Wbp1p showed high sensitivity to treatment with endo H in the same microsomal fractions, as deglycosylation led to a mobility shift in treated and mock digested samples. Unlike Wbp1p which appeared to be highly glycosylated, neither potential glycosylation site within the native protein sequence of Erg1p was efficiently glycosylated.

Discussion

Squalene-epoxidase, encoded by *ERG1*, has been shown to play an essential role in the biosynthetic pathway leading to the formation of ergosterol. Its dual localization, but at the same time exclusive restriction of activity to the ER raised the question as to the purpose of this limitation. Understanding the three-dimensional structure of Erg1p will help to unravel this discrepancy. High resolution structural information for Erg1p are not available at present.

Nevertheless, Ruckenstuhl *et al.* (Ruckenstuhl *et al.*, 2007) presented a homology based model of Erg1p that was built on the basis of the crystal structure of PHBH (*p*-hydroxybenzoate hydroxylases from *Pseudomonas fluorescens*). Unfortunately, the last 50 residues of the C-terminal end of Erg1p which have been shown before to be important for LP localization (Müllner *et al.*, 2004), could not be modelled, as PHBH does not show corresponding parts.

Differences in the membrane topology of Erg1p might give a reasonable explanation for functionality of this enzyme. Based on topology analysis of Erg1p we aimed to find a general mechanism how proteins may get embedded in the LP phospholipid monolayer. One feature most of the LP proteins have in common is the presence of one or several highly hydrophobic stretches. Proteolysis approaches using proteinase K revealed that both the N- and C-terminus of Erg1p from LP are facing the cytosol. The idea that longer domains of the protein could protrude into the highly hydrophobic core of the LP is unlikely as such areas need to be extremely hydrophobic over a long distance. The amino acid positions 138 and 380 which have been marked with small tags were also localized on the LP surface facing the cytosol. Only one site of Erg1p that was investigated within this study could not be assigned to a cytosolic orientation. The natively occurring recognition site for the specific protease fXa at aa-position 195 appears to be at least in part protected from the proteolytic treatment. Preliminary results using natively occurring potential glycosylation sites, revealed that the glycosylation sites at aa-position 159 and 166 are also oriented to the cytosol. Efficient glycosylation is not only dependent on the availability of specific recognition sequences but as well on proper spacing of the acceptor site in the luminal loop of the membrane protein from the membrane surface (Wessels and Spiess, 1988; Olender and Simon, 1992). Thus, only actually occurring glycosylation events and subsequent treatment with endo H will lead to reliable results.

Attempts to map the topology of Erg1p on biological membranes are so far too speculative. Especially the fact that LP only possess a phospholipid monolayer makes interpretations of any kind complicated. Computer predictions of membrane proteins are restricted to phospholipid bilayes and are therefore purely speculative. Computationally calculated data obtained with Erg1p preferentially suggest that both N- and C-termini face the cytosol and a large part of the protein is embedded within the membrane. The data and interpretations presented above will serve as the basis for further topology studies regarding LP proteins. The usage of specific proteases and tagging of internal sites seem to be the most promising methods. To continue the strategy with protease fXa, the native recognition site needs to be “disarmed”. The removal of this site was already achieved by using site-directed mutagenesis. Further cleavage sites will have to be inserted. The choice for the site of insertion will be of crucial importance. Additionally, glycosylation scanning will be another prospective approach, although this method will be restricted to elucidate the topology of Erg1p in the ER. In contrast to inserting internal tags, the insertion of artificial sites of glycosylation has a big advantage. The mutations necessary to remove endogenous sites and the resulting absence of glycosylation are not expected to have profound effects on enzyme activity and the topology of membrane proteins (van Geest and Lolkema, 2000). Combination of several approaches and covering long distances of the protein will lead to elusive data and clarification of the protein topology on both biological membranes.

Acknowledgments

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Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source
BY4741	*Mata lys2*Δ0 *leu2* Δ0 *ura3* Δ0 *his3*Δ1	EUROSCARF
ATCC 201388 Erg1p-TAP	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ERG1-TAP::HIS3MX6*	Open biosystems
KLN1	*Mat*α *leu2Δ0 ura3Δ0 trp1Δ1 ERG1::URA3*	Landl K. et al., 1996

Table 2. Plasmids

Plasmids	Description	Source
pUP2	pUG36 with GFP-*ERG1*	Potocnik U., 2005
pUP4	pUG35 with *ERG1*-GFP	Potocnik U., 2005
pESC-LEU-*ERG1*-414HA	*Bam*HI-*Sal*I fragment of ERG1 with HA tag at aa-position 138	This study
pESC-LEU-*ERG1*-1140HA	*Bam*HI-*Sal*I fragment of ERG1 with HA tag at aa-position 360	This study
pYES2-*ERG1*-fXaZero	*ERG1*p in pYES2 with I192V and G194A	This study

Table 3. Primers used for synthesizing *ERG1* variant with internal HA epitope
Underlined sequence represents HA epitope, bolded sequence encodes restriction sites for cloning

Construct		Fragment 1	Fragment 2
ERG1-414-HA	Fwd	5´ - CGC**GGATCC**ATGTCTG CTGTTAACGTTGCACCT – 3´	5´ - TACCCATACGACGTCCCAGACTAC GCTGATGATGAAAGAGAAAGGGGTGTT – 3´
	Rev	5´ - AGCGTAGTCTGGGACGT CGTATGGGTATTCGTAATCCT TGATGTGAATAGT – 3´	5´ - ACGC**GTCGAC**ACCAATCAACTCACC AAACAAAAATGG – 3´
ERG1-1140-HA	Fwd	5´ - CGC**GGATCC**ATGTCTGCT GTTAACGTTGCACCT – 3´	5´ - TACCCATACGACGTCCCAGACTACG CTCATTTCGAAAGAAAGAGTTACGAT – 3´
	Rev	5´ - AGCGTAGTCTGGGACGTC GTATGGGTAGTAGTCTAGTAA TTCATCCAAAAC – 3´	5´ - ACGC**GTCGAC**ACCAATCAACTCACC AAACAAAAATGG – 3´

Figures

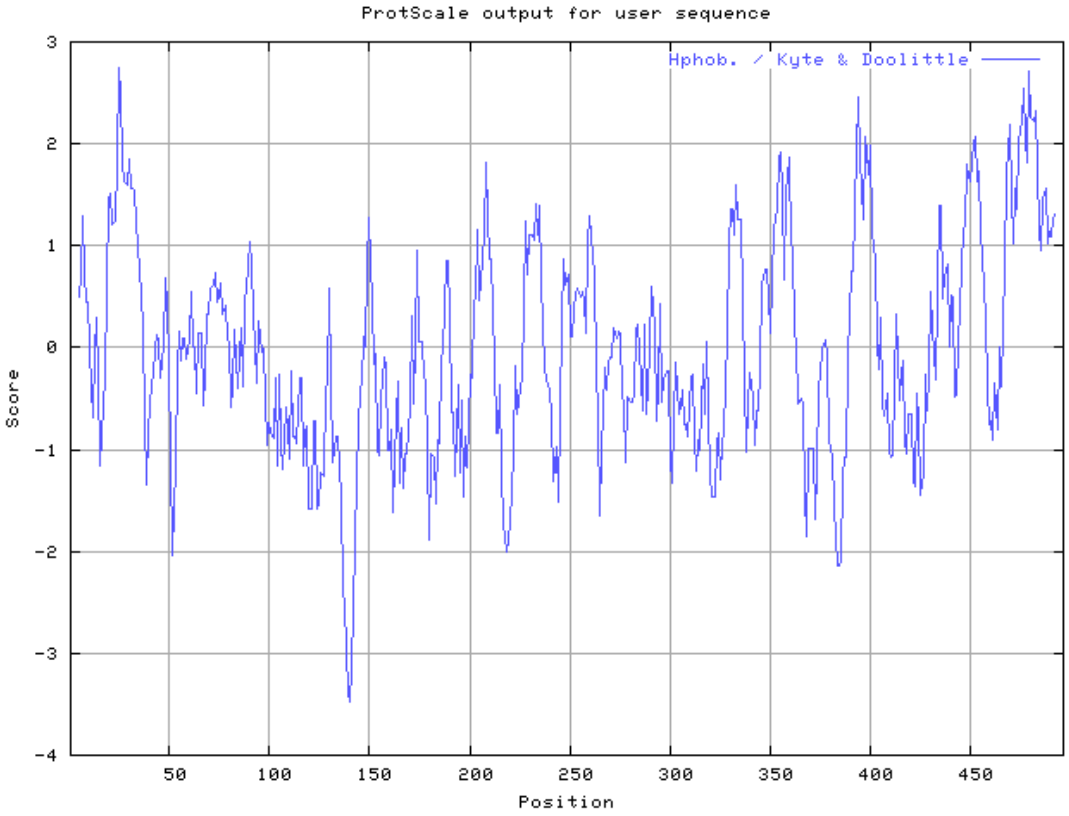

Figure 1

Hydropathy Plot of Erg1p.

In this Kyte-Doolittle plot hydrophobic residues are shown above zero, whereas the hydrophilic residues are below zero.

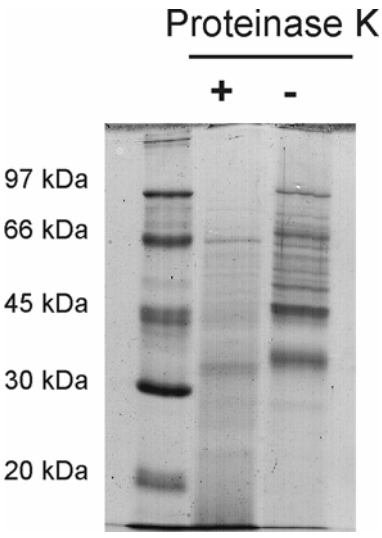

Figure 2

Proteinase K treatment of wild type LP.

LP of wild type BY4741 were treated with proteinase K. After stopping the proteolysis proteins were analyzed by SDS-PAGE.

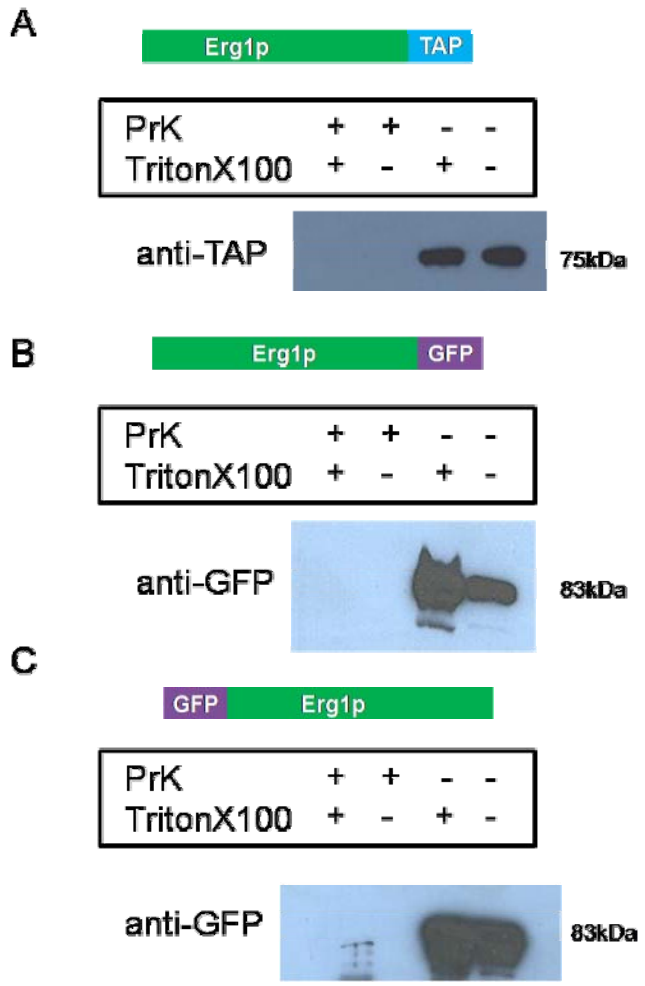

Figure 3

The N- and C-termini of Erg1p are located in the cytosol.

A, LP of strain ATCC, having TAP-tag fused to the C-terminal end of wild type Erg1p, were treated with proteinase K or mock-digested. B, LP of strain KLN1 carrying plasmid pUP2 expressing wild type Erg1p tagged to GFP at the C-terminus were treated with proteinase K or mock-digested. C, LP of strain KLN1 carrying plasmid pUP4 expressing wild type Erg1p tagged to GFP at the N-terminus were treated with proteinase K or mock-digested. Treatment with proteinase K was done in absence and presence of Triton X-100 (0.4%). Terminal tags were detected with either antisera against TAP (top) or GFP (middle, bottom).

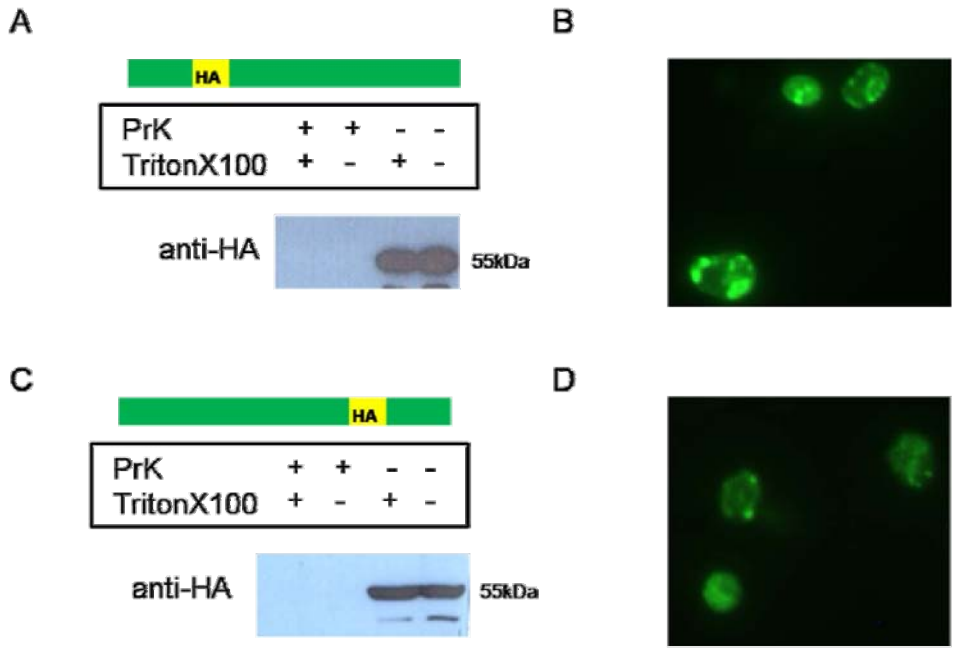

Figure 4

Internal positions within Erg1p are located in the cytosol.

A, Proteolytic digestion or mock-digestion with proteinase K of LP from strain BY4741 carrying plasmid pESC-LEU-*ERG1*-414-HA in the absence or presence of TritonX-100. HA epitope was detected with anti-HA. B, Indirect immunofluorescence shows that ERG1-414-HA localized to LP. Cells were prepared as described in Materials and Methods, and immunoreactive proteins were detected with Goat anti-mouse IgG, DyLight^{TM}488 secondary antibody. Primary antibody against HA was used. C, Proteolytic treatment or mock-digestion with proteinse K of LP from strain BY4741 carrying plasmid pESC-LEU-*ERG1*-1140-HA in the absence or presence of Triton X-100. D, Indirect immunofluorescence microscopy of *ERG1*-1140-HA.

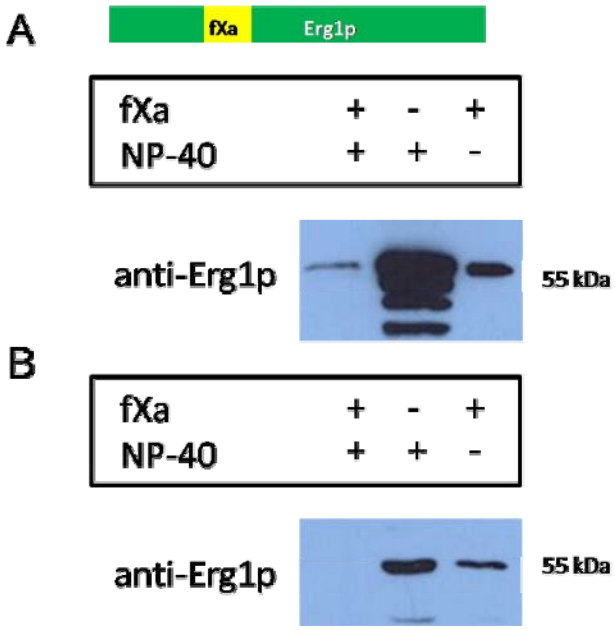

Figure 5

Factor Xa protease treatment of LP and ER.

A, Treatment of LP from wild type cells BY4741 with protease fXa in the absence or presence of detergent. B, Proteolysis of 40,000 g microsomes from wild type cells BY4741 with protease fXa in the absence or presence of detergent. In both cases, immunodetection was conducted with antibody against Erg1p.

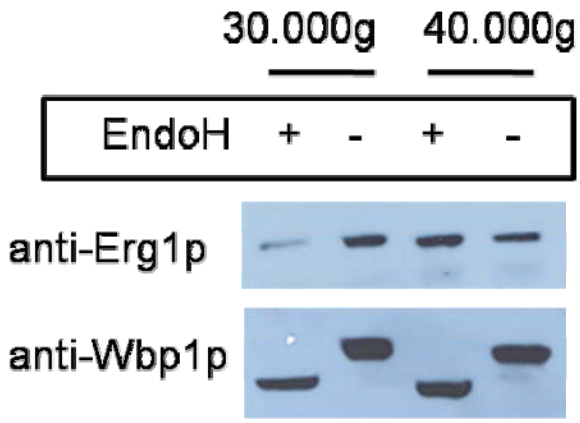

Figure 6

Glycosylation status of native Erg1p.

30,000 g and 40,000 g microsomes of wild type cells BY4741 were analyzed regarding the Erg1p and Wbp1p glycosylation status. Microsomes were treated or mock-digested with endoglycosidase H. Proteins were separated by 12.5% SDS-PAGE and proteins detected using anti-Erg1p and anti-Wbp1 serum.

General Discussion

Importance of lipid homoeostasis

Artherosclerosis, diabetes, obesity and certain neurodegenerative conditions are only a few serious lipid related diseases that affect our society in a cumulative manner. Disruption of processes related to lipid metabolism such as lipid synthesis, transport or storage might result in diseases mentioned above. Sterols and fatty acids are pivotal players in maintaining lipid homoeostasis in all kind of cells. Lipid molecules are important components as fatty acids for example provide a valuable chemical energy source and in addition serve as building blocks for membranes (Garbarino and Sturley, 2005). Sterols are as well indispensable constituents of eukaryotic membranes as their planar structure confers rigidity to membranes (Henneberry and Sturley, 2005). Besides their structural properties sterols and fatty acids are well known to act as effective signalling molecules and regulate gene expression by means of upstream promoter elements, such as OREs (oleate response elements) (Duplus *et al.*, 2000; Trotter, 2001) and SREs (sterol response elements) (Shimano, 2001). When sterols and fatty acids accumulate within the cell above average levels, these excess lipids can exert a cytotoxic effect. The yeast *Saccharomyces cerevisiae* reacts instantly with “detoxification” of the perturbing substrates. One way of reaction is the conversion of fatty acids and sterols into the biological inert form of neutral lipids which at the same time conserve the chemical energy but are not suited to be incorporated into biological membranes. The neutral lipids steryl esters (STE) and triacylglycerols (TAG) are formed and segregated into the two leaflets of the endoplasmic reticulum (ER) by two STE-Synthases (Are1p and Are2p) and two TAG-Synthases (Dga1p and Lro1p). Combined action of these enzymes leads to accumulation of neutral lipids resulting in the formation of a very hydrophobic droplet-like structure that will bud off the ER once a certain size is exceeded, forming an independent subcellular organelle,

the lipid particle (LP). Stored neutral lipids in the yeast can be used as substrates to become "recycled" by the action of STE-hydrolases (Yeh1p, Yeh2p and Tgl1p) and TAG-lipases (Tgl3p, Tgl4p and Tgl5p). How these proteins that are partially localized to the LP get access to their substrates has not been elucidated so far. Esterification of sterols and free fatty acids and hydrolysis of STE and TAG from LP play a crucial role in cellular sterol homeostasis (see Chapter 1).

Mobilization of STE

A pool of STE that is able to adapt to the cellular requirements is a valuable tool for the cell to protect itself against both a surplus and a lack of free sterols (Brown *et al.*, 1980). Therefore, enzymes capable of hydrolyzing STE are important to maintain cellular sterol levels. Yeast possesses three STE hydrolytic enzymes, Tgl1p, Yeh1p and Yeh2p with the first two localized to the LP and the last found in the plasma membrane (Taketani *et al.*, 1978; Köffel *et al.*, 2005). The highest STE hydrolase activity was found in the plasma membrane (Zinser *et al.*, 1993), whereas under anaerobic conditions the action of Yeh1p, which is localized to the LP becomes essential for proper mobilization of STE under these conditions (Köffel and Schneiter, 2006). Results obtained and described in Chapter 2 confirmed that the three hydrolases (see above) are most likely the only enzymes of this kind in yeast. Analysis of biochemical properties of these enzymes revealed different specificities for substrates. Spatial separation of enzymes with overlapping functions that are complementary in their substrate specificity could be part of a regulation mechanism providing proper and efficient STE hydrolysis. By which mechanisms the hydrolytic enzymes get access to their substrate is still a matter of dispute. It is very likely that these enzymes are regulated, because otherwise uncontrolled hydrolysis of the stored substrates would be the result.

Modulation of sterol homeostasis by Ste20p and Cla4p

All cell types polarize, at least transiently during division or to generate specialized shapes and functions. This capacity extends from yeast to mammals. In eukaryotic cells signal transduction and polarity are regulated by Cdc42p. Throughout the life cycle in the yeast *Saccharomyces cerevisiae* this Rho-type GTPase triggers different types of polarized growth in order to establish and maintain cell polarity (Johnson, 1999; Etienne-Manneville, 2004). Among the Cdc42p effectors that regulate cell polarization are Ste20p and Cla4p, both members of the p21-activated kinase (PAK) family which promote various processes of cytokinesis and cell cycle regulation (Park and Bi, 2007). Previously Tiedje C. *et al*. (Tiedje *et al.*, 2007) had shown that Ste20p binds to enzymes involved in sterol biosynthesis. Within Chapter 3 a possible influence of the Cdc42p effectors Ste20p and Cla4p on sterol metabolism is discussed. Cells lacking either one of the two Cdc42p effectors showed increased levels of sterols, and overexpression of *STE20* brought about lower sterol concentrations suggesting an inhibitory function of the two effectors on sterol biosynthesis. Further evidence was found that Cla4p has a negative effect not only on sterol biosynthesis but also on STE formation. In contrast, neither *STE20* deletion nor overexpression had any effect on STE levels or STE synthase activity. Additionally Ste20p and Cla4p as well down-regulate sterol uptake by inhibiting expression of genes involved in this process (Lin and Höfken, manuscript submitted). Therefore, it seems that Ste20p and Cla4p negatively influence several important sterol homeostatic processes. By influencing sterol homeostasis Ste20p and Cla4p can in part contribute to cell polarization. The discussed effectors could very well link STE mobilization with different stages of the cell cycle. This process was previously described for Tgl4p, a yeast TAG lipase, which gets activated by Cdc28p (Kurat *et al.*, 2009).

Effect of oleic acid on molecular composition of LP

The pattern of fatty acids in yeast is rather simple. Approximately 80% of yeast fatty acids are monounsaturated fatty acids consisting mostly of C16 and C18 fatty acids. The unsaturated fatty acids in *Saccharomyces cerevisiae* are produced from saturated fatty acyl CoA precursors by Ole1p, the only yeast fatty acid desaturase, which is localized to the ER. Although *Saccharomyces* is able to produce all required fatty acids de novo, it can as well import a wide range of exogenous long chain saturated and polyunsaturated fatty acids from the medium (Martin *et al.*, 2007). In this Thesis, yeast cells were incubated with the monounsaturated oleic acid and LP analyzed for their molecular composition of single compounds. Growth on oleic acid resulted in several major changes. Besides the induction of proliferation of peroxisomes, amount and size of LP were also strongly increased. The general pattern of single phospholipids was not much affected compared to wild type, whereas the pattern of neutral lipids was dramaticaly changed. Under normal conditions balanced levels of TAG and STE can be found. Cultivation of cells with oleic acid led to a strong increase in TAG at the expense of STE with oleic acid being the major fatty acid esterified in TAG. Some phospholipids of yeast LP were more affected than others by oleic acid. The most moderate changes were observed for phosphatidylinositol. In general, a pronounced shift towards the incorporation of oleic acid could be seen which led to a strong reduction in the appearance of saturated fatty acids. Again, phosphatidylinositol was the exception and did not show a reduction as pronounced as the other major phospholipids of the LP. It can be assumed that enzymes of phospholipid biosynthesis pathways show different specificities towards their substrates. When excessive amounts of free fatty acids, in our case oleic acid, is exogenously available cells rapidly take up these components from the exterior and incorporate them into membrane phospholipids and other lipids. In addition, oleic acid appears to have signalling functions. Besides its binding to the oleate respond elements (ORE) oleic acid appears to have

a direct inhibitory effect on STE synthesizing enzymes (Connerth et al., manuscript in preparation).

Orientation of LP resident Erg1p

Irrespective of their high lipid content, LP contain a distinct set of proteins. These proteins are known to be specialized for lipid biosynthesis, storage and mobilization of lipids and involved in intracellular membrane traffic (Zweytick *et al.*, 2000; Brasaemle *et al.*, 2004; Binns *et al.*, 2006). Mammalian lipid droplets and plant oil bodies are analogues of the yeast LP. In contrary to yeast LP characteristic structural proteins of the corresponding plant and mammalian organelles are known. As example, perilipin coats the entire lipid droplet in mammalian cells thus inhibiting lipases to get access to the substrates. Upon stimulation, perilipin becomes phosphorylated and enables degradation of lipids (Londos *et al.*, 2005). No such mechanism has so far been shown with yeast. Plant oleosins and mammalian perilipins contain hydrophobic sequence regions which are specialized for a proper anchoring of the protein in the monolayer of the organelle. Specialized domains such as the PKN (proline knot motif) in oleosins (Abell *et al.*, 2004) or polar residues (Ingelmo-Torres *et al.*, 2009) flanking the hydrophobic stretches seem to assist anchoring of the proteins. Yeast LP as well contain hydrophobic stretches (Müllner *et al.*, 2004). Despite the knowledge of specific sequence requirements no information about the topology of proteins that localize to the LP was obtained so far. Chapter 6 of this work is a preliminary approach to map the topology of the yeast model protein Erg1p, which is one of the most prominent LP proteins. Application of proteolytic approaches in combination with utilization of various tags and immunofluorescence yielded preliminary results. Both N- and C-termini are located towards the cytosol and the majority of the protein appears to be outsided the LP surface membrane. So far only one approach resulted in the finding of a short sequence that seemed to be

protected by the membrane. The topology of LP proteins is of crucial interest as it might help to explain how proteins are anchored, get access to their substrate or some enzymes are inactive when stored at the LP.

Conclusion

During the last decade major progress has been made in the understanding of lipid storage. It has been shown that lipid depots are not inert but rather affect through their flexible formation and mobilization a number of cellular processes. The LP which plays a central role in neutral lipid homeostasis has gained much attention. Nowadays, LP is sometimes regarded as an independent organelle, sometimes also as a side branch of the ER due to its function and biogenesis. The Thesis presented here has shed some light on the molecular properties of LP components, both the lipids and the proteins. The high flexibility of LP composition is remarkable on one hand, but on the other hand changes in the LP caused unexpected wide-ranging effects in cell structure and metabolic regulation. Conclusively, it appears that we are only at the beginning to understand links within the network formed around the process of lipid storage.

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List of publications

- Lin M, Grillitsch K, Daum G, Just U and Hoefken T. (equally contributed) Regulation of sterol synthesis and steryl ester formation by cell polarity proteins in budding yeast. FEBS J. 2009. 276(24):7253-64.
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- Rajakumari S, Grillitsch K, Daum G. (equally contributed). Synthesis and turnover of non-polar lipids in yeast. Prog Lipid Res 2008. 47(3):157-71.

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Poster presentations / oral presentations

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Structural analysis of lipid particles from the yeast Saccharomyces cerevisiae
September 4-8, 2007, Turku, Finland (48th International Conference on the Bioscience of Lipids)

Grillitsch K., Connerth M., Rietschel B., Karas M., Köfeler H. and Daum G.
Yeast lipid particles: lipidome meets proteome
December 13-17, 2008, San Francisco, USA (48th Annual Meeting of the American Society for Cell Biology)

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