Janus-faced Enzymes Yeast Tgl3p and Tgl5p Catalyze Lipase and A cyltransferase Reactions

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In the yeast, mobilization of triacylglycerols (TAGs) is facilitated by the three TAG lipases Tgl3p, Tgl4p, and Tgl5p. Motif search analysis, however, indicated that Tgl3p and Tgl5p do not only contain the TAG lipase motif GXGGG but also an H-(X)_{n}-D acyl transferase motif. Interestingly, lipid analysis revealed that deletion of TGL3 resulted in a decrease and overexpression of TGL3 in an increase of glycerophospholipids. Similar results were obtained with TGL5. Therefore, we tested purified Tgl3p and Tgl5p for acyltransferase activity. Indeed, both enzymes not only exhibited lipase activity but also catalyzed acylation of lysophosphatidylethanolamine and lysophosphatidic acid, respectively. Experiments using variants of Tgl3p created by site-directed mutagenesis clearly demonstrated that the two enzymatic activities act independently of each other. We also showed that Tgl3p is important for efficient sporulation of yeast cells, but rather through its acyltransferase than lipase activity. In summary, our results demonstrate that yeast Tgl3p and Tgl5p play a dual role in lipid metabolism contributing to both anabolic and catabolic processes.

INTRODUCTION

Phospholipids are major components of cellular membranes that actively take part in a series of metabolic events including maintenance of the cellular permeability barrier, regulation of the activities of proteins associated with the membrane and regulation of intracellular signaling by serving as precursors of signaling molecules (Dowhan, 1997; Yamashita et al., 1997; Voelker, 2000, 2005; Gijón et al., 2008). There are two major pathways for de novo formation of phospholipids, namely, the cytidine diphosphate (CDP)-choline/CDP-ethanolamine pathway, and the de novo CDP-diacylglycerol (DAG) pathway (Kennedy and Weiss 1956; Kent 1995; Bürgermeister et al., 2004; Rosenberger et al., 2009). In eukaryotic cells, phosphatidic acid (PA) is a central precursor molecule for the synthesis of major glycerophospholipids and nonpolar lipids (Carman and Henry, 2007). In the de novo pathway, PA is synthesized from glycerol 3-phosphate and then used for the synthesis of glycerophospholipids with other head groups through the CDP-DAG pathway (Athenstaedt and Daum, 1999). PA can also be dephosphorylated to DAG and used for the synthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) via the CDP-ethanolamine and CDP-choline branches of the Kennedy pathway (de Kroon, 2007). Moreover, DAG derived from PA is also used for the synthesis of triacylglycerol (TAG) (Rajakumari et al., 2008). It was shown that PA can also be produced from PC and PE by phospholipase D (PLD), and DAG from the various phospholipids by catalysis of phospholipase C. Besides pathways described above glycerophospholipids can also be generated by acylation and deacylation process called Land’s cycle or phospholipid remodeling (Lands, 1960; Kennedy, 1961; Lands and Merkl, 1963; Merkl and Lands, 1963) in which the rapid turnover of the sn-2 acyl moiety of phospholipids is carried out by phospholipase A_{2} and lysophospholipid (LPL) acyltransferases.

The possible involvement of LPL-acyltransferases in phospholipid biosynthesis and remodeling has led to the identification of some enzymes of this type in mammalian system such as lysophosphatidic acid (LPA) acyltransferase (LPAAT) and lysophosphatidylcholine (LPC) acyltransferase (LPCAT) (Chen et al., 2006; Zhao et al., 2008). In the yeast, the gene product of SLC1 was identified as LPAAT, contributing ≈60% to the cellular enzymatic activity (Nagiec et al., 1993; Athenstaedt and Daum 1997). Recently, another enzyme of this type named Slc4p, Lpt1p, or Ael1p was identified independently by different research groups (Benghezal et al., 2007; Jain et al., 2007; Riekhof et al., 2007; Tamaki et al., 2007). This enzyme was shown to be involved in lyso-PE (LPE) acylation. Deletion of ALE1/LPT1/SLC4 strongly reduced the LPE acyltransferase (LPEAT) activity in the yeast microsomal fraction. Both acyltransferases, Slc1p and Ael1p/Lpt1p/Slc4p, belong to the family of membrane-bound O-acyltransferases (Hofmann, 2000). Investigations with another acyltransferase family, the glycerol-3-phosphate acyltransferases (GPAT) or LPAAT, revealed that four conserved domains seem to be responsible for the catalytic function of these types of enzymes (Heath and Rock, 1998). Acyltransferase motifs included the sequences H-(X)_{n}-D (motif I), GVIFIDR (motif II), EGTR (motif III), and IVPIVM (motif IV). Among these motifs, acyltransferase motifs I and III are best conserved by function (Dircks et al., 1999; (Lewin et al., 1999; Leung, 2001) in acyl-CoA:diacylglycerol acyltransferase and 2-acylglycerophosphati-
Table 1. Strains used in this study

| Strain                  | Genotype                                      | Source                  |
|-------------------------|-----------------------------------------------|-------------------------|
| BY4741 wild-type        | Matα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0         | Euroscarf collection    |
| tgl3Δ tgl5Δ             |                                               | Atherstaedt and Daum    |
| FY1679                  | MATA; ura3-52, trp1Δ163, leu21, his3200       | Euroscarf collection    |
| TGL3-His6               | plasmid pFA6a-TRP-PLG1L for N-terminal tagging| This study              |
| BY4743 2n               | MATA/MATA; his3Δ1; leu2Δ0; trp1Δ163; met15Δ0;| Euroscarf collection    |
|                        | lys2Δ0; his3Δ1; leu2Δ0; trp1Δ163; met15Δ0;    |                         |
|                        | lys2Δ0; his3Δ1; leu2Δ0; trp1Δ163; met15Δ0;    |                         |
|                        | YMR313c::kanMX4                               |                         |
|                        | YKR089c::kanMX4                               |                         |
| GS115                   | his4                                          | Invitrogen              |
| Tgl5p-His6              | GS115, TGL5-His6 tag at the N terminus        | This study              |
| tgl3Δ                   | MATα, ura3-52, trp1Δ163, leu2Δ1, his3Δ200, Δtgl3::kanMX4| Euroscarf collection    |

**Materials and Methods**

**Strains and Culture Conditions**

Yeast strains used throughout this study are listed in Table 1. Cells were grown in YPD (1% yeast extract, 2% bacto peptone, and 2% glucose) or synthetic minimal media (SM) containing 0.67% yeast nitrogen base (Difco, Detroit, MI) supplemented with the appropriate amino acids and 2% glucose or 2% galactose, respectively. For heterologous expression of His-tagged Tgl3p, *V. parietina* cells were grown on buffered minimal methanol medium (BMM10) containing 1.34% yeast nitrogen base, 4 × 10⁻³% β-glucuronidase (Sigma-Aldrich, St. Louis, MO), 5% methanol, and 200 mM potassium phosphate, pH 6.5. For sporulation of *S. cerevisiae*, medium containing 2% potassium acetate, 0.1% yeast extract, and 0.05% raffinose or 0.05% galactose, respectively, were inoculated with a colony of a diploid strain grown from solid medium and shaken at 32°C.

**Bioinformatics Analysis**

Conserved protein domains and motifs were examined using the CDD at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and Pfam database (http://pfam.sanger.ac.uk) as described previously (Bateman et al., 2000).

**Microscopy**

Sporulation efficiency was monitored by fluorescent microscopy. Cells were fixed in ethanol and stained with 4,6-diamidino-2-phenylindole (DAPI) as described previously (Rose et al., 1995). Yeast spores were inspected using an Axiosview 35 microscope (Carl Zeiss, Jena, Germany), with a 100-fold oil immersion objective.

**Site-directed Mutagenesis**

Plasmid pYES2TGL3 was obtained as described previously by Atherstaedt and Daum (2003). Plasmids pYES2TGL3 lots, pYES2TGL3 lots, and pYES2TGL3 lots were constructed using the QuickChange polymerase chain reaction (PCR)-based mutagenesis procedure (Stratagene, La Jolla, CA) with the pYES2TGL3 lots plasmid as template following the manufacturer's instructions. Primers used to construct site-directed mutants are listed in Table 2.

**Overexpression and Isolation of Tgl3p and Tgl5p**

Overexpression of His-tagged hybrids of Tgl3p under a galactose-inducible promoter in *S. cerevisiae* has been described by Atherstaedt and Daum (2003).
Table 2. Oligonucleotides for site-directed mutagenesis of TGL3

| Mutation | Oligonucleotide sequence |
|----------|--------------------------|
| S237A    | (+) 5'-CTAATATTTAAACGTTGACCCGCAATGGGGCATCGTTG-3' |
|          | (-) 5'-CAACGCCATGGCCTGGGCTGAATCTTATATTAG-3' |
| H298A    | (+) 5'-CT&TGATTTCAAGACCACACTACCCTATCTGACCTTAT-3' |
|          | (-) 5'-GATAAAGGCTTTGAAAGATGGCCCGGCTGAATTTCTGCTAACAA-3' |
| D303A    | (+) 5'-CCACGCAGTTATTTCAAGCCTATTCTTATTTATC-3' |
|          | (-) 5'-CCGATAAAGAAACCTCCTGAGAATAACCCGTTG-3' |
| D303E    | (+) 5'-CCGACCGTTATTTCAAGCAGTTATTTATC-3' |
|          | (-) 5'-CCGATAAAGAAACCTCCTGAGAATAACCCGTTG-3' |

Tgl5p was heterologously expressed in Pichia pastoris under the AOX1 promoter with 1% methanol as a carbon source as published by Athenaestadt and Daum (2005).

For the isolation of the His-tagged Tgl5p, lipid particles were isolated at high purity from yeast cells grown to the early stationary phase by the method of Leber et al. (1994). Lipid particle proteins were solubilized in the presence of 8 M 3-[3-hydroxypropylidene]dimethylammonio]propanesulfonate (CHAPS), and subsequently the bound protein was eluted with 5 ml of elution buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 250 mM imidazole, and 125 mM CHAPS). Fractions of 1 ml were collected and aliquots of 90 μl were used for measuring the acyltransferase activity as described above.

For the isolation of His-tagged Tgl5p, P. pastoris cells overexpressing the respective gene were grown in BMM10 for 24 h and harvested by low-speed centrifugation. Cells were suspended in 50 mM Tris-HCl, pH 7.4, 0.5 M sucrose, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Cells were disintegrated using glass beads, and unbroken cells and debris were removed by centrifugation at 3000 × g for 10 min. The cell-free extract was centrifuged at 100,000 × g for 90 min to obtain the cytosol in the supernatant, and the total membrane fraction in the pellet. The cytosolic fraction that contained the majority of the enzyme activity was used for Tgl5p purification as described above.

**Protein Analysis**
Protein measurements were performed by the method of Lowry et al. (1951) by using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (1970). Western blot analysis, 12.5% polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (1970). For the isolation of the His-tagged Tgl5p, lipid particles were isolated at high purity from yeast cells grown to the early stationary phase by the method of Leber et al. (1994). Lipid particle proteins were solubilized in the presence of 8 M 3-[3-hydroxypropylidene]dimethylammonio]propanesulfonate (CHAPS), and subsequently the bound protein was eluted with 5 ml of elution buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 250 mM imidazole, and 125 mM CHAPS). Fractions of 1 ml were collected and aliquots of 90 μl were used for measuring the acyltransferase activity as described above.

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**Metabolic Labeling of Phospholipids and Neutral Lipids**
The Tgl3p-His6−overexpressing strain and its corresponding wild type FY1679 were precultured in 5 ml of YPD containing 2% glucose. For in vivo labeling, cells at an OD of 0.2 were transferred to a fresh induction medium containing 2% galactose and 1 μCi/ml [14C]acetate (specific activity 51 mCi/mmol) and grown for additional 24 h. Cells (OD 10) were harvested by centrifugation, and lipids were extracted using chloroform/methanol (2:1; vol/vol). Lipids were separated by two-dimensional thin layer chromatography (TLC) on silica gel 60 using chloroform/methanol/water (65:35:5, per vol) as the solvent system. Individual lipids were separated by one-dimensional TLC using chloroform/methanol/acetic acid (70:30:1, per vol) as the solvent system. Individual lipids were visualized by exposure to iodine vapor. Lipid bands were identified by their relative migration compared with the standards. Radioactively labeled lipids were scraped off after removal of the iodine and quantified by a liquid scintillation counter (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences, Boston, MA) using LSC Safety (Malinckrodt Baker, Phillipsburg, NJ) plus 5% water as scintillation cocktail.

**Lipase, Phospholipase, and Transacylase Assays**
Lipase activity was measured in 100 mM Tris-HCl pH 7.5, containing 2 mM EDTA and 1 mM diethiothreitol. The substrate mixture containing 20 μM egg phospholipids (PC) and 100 μM [9,10-3H]triolein was combined in a tube and dried under a stream of nitrogen. Then, dried lipids were resuspended in the reaction buffer using 100 μM sodium taurocholate. Finally, the substrate was added in the form of sonicated vesicles or suspension to the total reaction volume of 200 μl. Lipids were extracted using 200 μl butanol followed by TLC using light petroleum/diethyl ether/acetic acid (70:30:1, per vol) as solvent system. Radiolabeled products were quantified by scintillation counting. Phospholipase and transacylase activities were measured according to Jenkins et al. (2004).

**RESULTS**
Overexpression of TGL3 Increases the Cellular Content of Phospholipids
Previous work from our laboratory had shown that deletion of TGL3 resulted in an accumulation of TAG with an altered fatty acid profile (Athenaestadt and Daum, 2003). In the present study, which was aimed at a more detailed biochemical and cell biological characterization of yeast TAG lipases, we first extended our analyses to the phospholipid profile of tgl3Δ deletion and TGL3 overexpressing strains. For the latter purpose, we used a strain that overexpressed a Tgl3p-His6 fusion protein under a GAL1 promoter. Overexpression of this polypeptide was confirmed by SDS-PAGE and Western blot analysis. Quantification of total phospholipids revealed that deletion of TGL3 resulted in a reduction (−34%) and overexpression of TGL3 in an increase (+37%) of total phospholipids compared with wild-type cells (Figure 1A). A more detailed analysis of phospholipids in these two strains (Figure 1B) showed that overexpression of TGL3 led to an increase of PS, PE, and PC by ~31, 37, and 33%, respectively, and deletion of TGL3 to a decrease of these three phospholipids to 25, 30, and 36%, respectively. In contrast, overexpression of TGL3 reduced the TAG level only by 12% compared with the wild-type control (data not shown). Therefore, the increased levels of PE and PC in the TGL3 overexpressing strain were most likely not only due to increased lipase activity. This result chal-

Vol. 21, February 15, 2010 503
Tgl3p Mediates Dual Function in Lipid Metabolism

The above-mentioned bioinformatics evidence led us to investigate whether the acyltransferase signature motif of Tgl3p indeed encodes for a catalytic function. For this purpose, we overexpressed N-terminal His₆-tagged Tgl3p, solubilized the hybrid protein from lipid particles, and purified it by affinity chromatography as described under Materials and Methods. The expression and isolation of the hybrid protein was confirmed by immunoblot using anti-His antibody and revealed a protein band at 73-kDa (Supplemental Figure S1). Because the Tgl3p overexpression increased the level of PC and PE in yeast (Figure 1) and contained a signature motif for acyltransferases (Figure 2), the obvious experiments to perform were lysophospholipid acyltransferase assays. Using [¹⁴C]oleoyl-CoA and various lysophospholipids as substrates, we were able to demonstrate that Tgl3p catalyzed acylation of lyso-PE in a very specific way (Figure 3A). Only minor activity was observed with LPC, LPA, and lyso-PS (LPS) as substrates. We also performed N-acyltransferase assays by using [³H]diacylglycerol as a substrate, but no detectable activity was observed (data not shown). As can be seen from Figure 3B, Tgl3p had a preference for the unsaturated substrate oleoyl-CoA (18:1). The enzymatic activity with other acyl-CoAs was minor.

The LPEAT reaction catalyzed by Tgl3p was time dependent with a linear range of 15 min and enzyme dependent with a linear range up to 400 ng protein under the specified assay condition. Enzyme kinetic measurements (Figure 3, C and D) revealed $K_M$ values of 19 ± 1.4 μM for lyso-PE and 18 ± 1.2 μM for oleoyl-CoA as substrates. The $V_{max}$ values were 44.25 ± 4.2 nmol/min/mg for oleoyl-CoA and 46.26 ± 3.2 nmol/min/mg for LPE, respectively. Control experiments using [³H]triacylglycerol or [³H]PC as cosubstrates instead of acyl-CoA indicated that Tgl3p did not catalyze transacylase reactions, whereas the TAG lipase activity of the isolated protein was confirmed (data not shown). These data demonstrated that PE was formed in an acyl-CoA dependent acylation of LPE and not via an acyl-CoA independent process. Because some acyltransferases were reported to be sensitive to specific ions, we tested LPEAT activity in the presence of various divalent cations. As examples, yeast LPCAT activity was remarkably inhibited by Zn²⁺ ion but insensitive to Mg²⁺, whereas LPAAT (Sic1p) activity was enhanced in the presence of Mg²⁺ (Benghezal et al., 2001; Lewin et al., 2008). It was suggested that motif I (H-(X₄)₄-D) is involved in acyl-CoA binding. In addition, the gene expression analysis in the S. cerevisiae Genome Database (SGD) showed that the TGL3 expression level was elevated during sporulation. Altogether, these in silico analyses supported our view obtained from in vivo experiments that Tgl3p might also act as an acyltransferase in yeast cells.

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sured was not due to the dual localization of Slc1p in lipid particles.

**Histidine Residues Are Indispensable for Acyltransferase Activity of Tgl3p**

To further confirm that Tgl3p has lysophospholipid acyltransferase activity in addition to its TAG lipase activity, we addressed the molecular details of this finding. We wanted to answer the question whether the potential acyltransferase motif of Tgl3p was indeed responsible for the observed reaction, or possible impurities of our preparations were the reason for these observations. The acyltransferase motif of Tgl3p comprises the sequence H-(X₄)-D (298HGYSQD303). To determine the functional significance of this conserved sequence in Tgl3p, we performed site-directed mutagenesis. We independently replaced each histidine residue by alanine (H298A), and also substituted D303E and D303A as described in Materials and Methods. In addition, the serine residue of the lipase motif was replaced by alanine (S237A). Using these variants of Tgl3p we examined acyltransferase and TAG lipase activities of enzymes overexpressed and purified. Figure 4 shows that all variants of Tgl3p were overexpressed at a comparable amount. Substitution of D303A and D303E resulted in a moderate reduction of acyltransferase activity, but the mutation H298A showed a more dramatic decrease of enzyme activity (Figure 4B). In contrast, mutation on S237A (TAG lipase motif) did not have any effect on acyltransferase activity. We also checked whether a mutation in the acyltransferase motif affected lipase activity. As can be seen from Figure 4B, alteration in the H-(X₄)-D motif did not affect the lipase activity, whereas S237A led to a marked reduction of this enzymatic activity. These results clearly demonstrated that the histidine residue

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**Figure 3.** Tgl3p mediates oleoyl-CoA dependent acylation of lysophosphatidylethanolamine. A Tgl3p-His₆ fusion protein was used to analyze enzyme activities. Acyltransferase assays were performed as described in Materials and Methods. (A) Purified Tgl3p was examined for the acyl acceptor specificity using LPA, LPC, LPE, LPI, and LPS as acceptors. (B) Acyl-CoA selectivity of purified Tgl3p. (C) Lineweaver-Burk plot for variable amounts of LPE used as substrate. (D) Lineweaver-Burk plot for variable amounts of oleoyl-CoA used as substrate. The mean values of three independent experiments ± SD are shown. NE, no enzyme; NS, no substrate.

**Figure 4.** Effect of amino acid substitution on lipase and acyltransferase activity of Tgl3p. Amino acid residues of the acyltransferase motif HGYSQD were replaced by site-directed mutagenesis as indicated. Mutagenized variants were overexpressed using a galactose inducible promoter, lipid particles were isolated, solubilized, and assayed for LPE acyltransferase activity. (A) Western Blot analysis of lipid particles from Tgl3p variants. (B) Enzymatic activity of Tgl3p variants. White bars, lipase activity; and black bars, acyltransferase activity. The values of three independent experiments ± SD are shown.
in the acyltransferase motif is important for this enzymatic activity, and acyltransferase and lipase motifs of Tgl3p act independently of each other.

**Overexpression of Tgl3p Rescues Delayed Log Phase of tgl3Δ tgl4Δ**

A previous report demonstrated that growth of a tgl3Δtgl4Δ double mutant was severely impeded in galactose medium and TAG mobilization was important for rapid initiation of growth (Kurat et al., 2006). To further authenticate the potential role of acyltransferase and lipase motifs of Tgl3p on cell growth, cells bearing S237A and H298A variants of the polypeptide in tgl3Δ tgl4Δ background were grown under the galactose-inducible GAL1/10 promoter. The expression of wild type and mutated forms of Tgl3p were confirmed by Western Blot analysis (data not shown). As shown in Figure 5, overexpression of pYES2-Tgl3pWT and pYES2-Tgl3pS237A rescued the delayed growth of tgl3Δ tgl4Δ. Alternatively, the H298A mutation in the acyltransferase motif of Tgl3p still showed the growth defect in galactose minimal medium. These data clearly indicate that rather the acyltransferase function of Tgl3p is essential to rescue the growth defect of tgl3Δ tgl4Δ than the lipolytic function of this enzyme.

**Acyltransferase Function of Tgl3p Is Essential for Sporulation**

Sporulation of *S. cerevisiae* is the process of gametogenesis which involves meiotic nuclear divisions and differentiation of a diploid mother cell into an ascus containing four haploid ascospores. Previous studies had described that during yeast sporulation gene expression profiles were extensively altered and specific sets of genes were highly expressed (Priming et al., 2000). As mentioned above, the Tgl3p and Tgl5p are maximally expressed during sporulation (www.yeastgenome.org), and homozygous tgl3Δ/tgl3Δ and tgl4Δtgl5Δ/tgl4Δtgl5Δ deletion mutants are defective in sporulation. This information led us to elucidate an apparent role of this gene during yeast sporation and to investigate whether lipase or acyltransferase functions of Tgl3p play a key role in this process. Hence, the homozygous diploid tgl3Δtgl3Δ mutant was transformed with empty pYES2 plasmid (vector control), pYES2-TGL3WT, pYES2-TGL3S237A and pYES2-TGL3H298A and the efficiency of spore formation was tested (Figure 6A). We observed that the tgl3Δtgl3Δ mutant carrying pYES2-TGL3WT and TGL3 bearing a mutation in the lipase motif (pYES2-TGL3S237A) sporulated as efficiently as the wild-type diploid strain, whereas the TGL3 variant with the point mutation in the acyltransferase motif (pYES2-TGL3H298A) exhibited a marked sporulation defect (Figure 6B). This experiment showed that TAG lipase activity of Tgl3p is dispensable, whereas the LPEAT activity is indispensable for yeast sporulation.

**Tgl5p Mediates Lysocephatidic Acid Acyltransferase Activity in Yeast**

Results obtained with the yeast TAG lipase Tgl3p as described above tempted us to speculate that the other two yeast TAG lipases described in the literature, Tgl4p and Tgl5p (Athenstaedt and Daum 2005; Kurat et al., 2006), may have similar properties. Tgl5p also has a typical patatin domain, which is crucial for acyltransferase activity. Thus, sequence motifs of Tgl5p resemble those of Tgl3p (Figure 2).
have an H-(X)\_2-D motif. Therefore, Tgl4p was not further investigated in the present study.

Here, we show that Tgl5p has similar properties as Tgl3p with respect to its capacity acting as an acyltransferase. Because the expression level of TGL5 is very low in S. cerevisiae (Athenstaedt and Daum, 2005; our unpublished data), we used P. pastoris to achieve high-level expression of a His-tagged version of Tgl5p from S. cerevisiae (see Materials and Methods) for functional characterization of this enzyme. Overexpression of TGL5 upon induction with methanol was confirmed by Western blot analysis using anti-His and anti-Tgl5p antibodies (data not shown). In vivo labeling with \[^{14}\text{C}\]\text{acetate demonstrated that overexpression of TGL5 led to an increase of total cellular phospholipids (Figure 7A), especially of PS and PI, PC, and PA (Figure 7B). The TAG level in this strain was decreased to ~60% of the control (Figure 7C).}

Functional Characterization of Tgl5p

For functional characterization, His\_tagged Tgl5p was purified by affinity column chromatography and subjected to enzymatic analysis. Using this enzyme source, lysophospholipid acyltransferase activity of Tgl5p was measured with various lysophospholipids as substrates (Figure 8A). These experiments clearly demonstrated that Tgl5p was specific for lyso-PA acylation, whereas only minor activities were detected with lyso-PC, lyso-PI, lyso-PE, and lyso-PS as substrates. Tgl5p showed time-dependent (linear range up to 15 min) and protein-dependent formation (linear range up to 600 ng) of radiolabeled PA under the specified assay conditions. Enzyme kinetic measurements revealed a $K_m$ value of 18.7 ± 1.5 μM and a $V_{max}$ value of 28.8 ± 2.4 nmol/min/mg for lyso-PA, and a $K_m$ value of 29.3 ± 1.2 μM and a $V_{max}$ value of 38.1 ± 3.1 nmol/min/mg for oleoyl-CoA as substrates (Figure 8A).

We further analyzed the acyl chain selectivity of Tgl5p by using \[^{1-^{14}\text{C}}\text{palmitoyl-CoA (16:0) or [1-^{14}\text{C}]oleoyl-CoA (18:1) as acyl donors. These acyl donors were chosen to distinguish between saturated and unsaturated fatty acids used for phospholipid synthesis. Tgl5p exhibited a clear preference for oleoyl-CoA (18:1) over palmitoyl-CoA (16:0) as the acyl donor (Figure 9A). During these enzymatic analyses, we also realized the sensitivity of Tgl5p to different detergents. The activity of the enzyme was strongly decreased in the presence of Triton X-100 (50% decrease at 0.5 mM), whereas the effect of CHAPS was much milder (50% decrease at 8 mM; Figure 9B). We further analyzed Tgl5p for possible other enzyme activities such as phospholipase, transacylase, and lysophospholipase activities. The purified protein, however, did not exhibit any of the above-mentioned activities (data not shown).

DISCUSSION

In the yeast, TAG metabolism is governed by the three patatin domain-containing lipases Tgl3p, Tgl4p, and Tgl5p, which are localized to lipid particles (Athenstaedt and Daum, 2003, 2005). In vegetatively growing cells, single deletions of the respective genes do not cause obvious phenotypic defects. During sporulation, however, TAG lipases are expressed at high level and sporulation efficiency is impaired in a tgl3Δ/tgl3Δ deletion mutant. So far, the function of TAG lipases in yeast sporulation has not been investigated at the molecular level. Our results presented here suggest that TAG lipases are directly involved in membrane lipid biosynthesis during sporulation and also in the early log phase of yeast cell growth. We ascribe this function to the sensitivity of Tgl3p and Tgl5p to different detergents. The activity of the enzyme was strongly decreased in the presence of Triton X-100 (50% decrease at 8 mM; Figure 9B). We further analyzed the acyl chain selectivity of Tgl5p by using \[^{1-^{14}\text{C}}\text{palmitoyl-CoA (16:0) or [1-^{14}\text{C}]oleoyl-CoA (18:1) as acyl donors. These acyl donors were chosen to distinguish between saturated and unsaturated fatty acids used for phospholipid synthesis. Tgl5p exhibited a clear preference for oleoyl-CoA (18:1) over palmitoyl-CoA (16:0) as the acyl donor (Figure 9A). During these enzymatic analyses, we also realized the sensitivity of Tgl5p to different detergents. The activity of the enzyme was strongly decreased in the presence of Triton X-100 (50% decrease at 0.5 mM), whereas the effect of CHAPS was much milder (50% decrease at 8 mM; Figure 9B). We further analyzed Tgl5p for possible other enzyme activities such as phospholipase, transacylase, and lysophospholipase activities. The purified protein, however, did not exhibit any of the above-mentioned activities (data not shown).

acetyltransferases in addition to their role as TAG lipases. In the present study, we demonstrate by experiments in vivo and in vitro that Tgl3p acylates LPE to PE with some preference. In addition, we reported that the purified TAG lipase Tgl5p acylates LPA to PA using oleoyl-CoA as acyl donor.

In the yeast, Slc1p and Slc4p/Ale1p/Lpt1p have been identified as most prominent lysophospholipid acetyltransferases, and an slc1Δ slc4Δ double deletion was shown to cause synthetic lethality in minimal media (Benghezal et al., 2007; Jain et al., 2007; Riekhof et al., 2007). Both enzymes exhibit a broad substrate specificity, i.e., they acylate more or
less all lysophospholipid. Therefore, it was not surprising
that overexpression of Tgl3p in the slc1/H9004 slc4/H9004
double mutant background did not rescue the synthetic lethal pheno-
type (data not shown). Moreover, Slc1p has Mg2+-depen-
dent LPAAT activity, whereas purified Tgl3p was Mg2+
independent in vitro and Cu2+ and Zn2+ severely inhibited
the LPEAT activity. We also observed that purified Tgl3p
has a preference for unsaturated acyl-CoA as cosubstrate.

Previous reports (Listenberger et al., 2003; Kohlwein and
Petschnigg, 2007; Kurat et al., 2009) demonstrated that phos-
pholipid synthesis is efficient during cell budding, and yeast
cells mobilize TAG thereby generating an acyl-CoA pool for
membrane lipid biosyntheses during exit from the station-
ary phase to the lag phase. Our results presented here sug-
test that Tgl3p and Tgl5p may specifically use this acyl-CoA
pool for the synthesis of membrane phospholipids via acyl-
CoA–dependent lysophospholipid acylation. This view is
supported by the finding that the growth defect of a tgl3Δ
tgl4Δ double deletion strain (Figure 5B) was specifically
rescued by overexpression of the Tgl3pH237A variant (acyl-
transferase active) but not by Tgl3pH298A (acyltransferase
inactive). This result illustrated that the acyltransferase but
not the TAG lipase function of Tgl3p is essential for mem-
brane lipid biosynthesis during the lag phase.

During sporulation, the meiotic progression is accompa-
nied by the formation of a prospore membrane which re-
quires membrane lipid synthesis. In general, phospholipases
and lipases play critical roles in cell signaling by producing
second messenger lipids such as DAG, PA, and lysophos-
pholipids. In line with these findings, it has been reported
that production of PA by the phospholipase D (Spo14p) is
indispensable for yeast cell sporulation (Ktistakis et al.,
1996). The N-terminal region of the enzyme was shown to be
important for proper localization of the polypeptide to the
developing membrane and membrane trafficking (Honig-
berg et al., 1992; Rose et al., 1995; Rudge et al., 2004). Expres-
sion analysis data from SGD (www.yeastgenome.org) also
indicates that expression levels of TGL3, TGL5, and TGL4 are
increased during sporulation that is in line with the obser-
vation that homologous diploid strains of tgl3Δtgl3Δ and
tgl4Δtgl5Δtgl4Δtgl5Δ double deletion strains bear a sporula-
tion defect (Athenstaedt and Daum, 2005). Our analysis

toward the dual functions of TAG lipases upon yeast sporu-
lation revealed that Tgl3p through its function as PE forming
acyltransferase is required for proper spore formation (Fig-
ure 6). Even though Tgl5p is not an efficient lipase compared with Tgl3p, it may act quite efficiently as LPAT during sporulation. Synthesis of PE and PA through the action of Tgl3p and Tgl5p may even play a key role in maintenance and maintaining the structural integrity of prosopese membranes. Finally, Tgl3p and Tgl5p may through their function as phospholipid biosynthetic enzymes even supply substrates to various phospholipases which in turn may be involved in membrane remodeling. During spore formation PLD activation is directly involved in membrane trafficking (Ktistakis et al., 1996) and reorganization of cytoskeleton (Cross et al., 1996). Similar to PLD activation, TAG lipases may also be activated via kinases (Kurat et al., 2009) and participate in membrane trafficking. This is, however, pure speculation at this stage of our knowledge. Conversely, our data clearly demonstrate that TAG lipases are not only hydrolyses, but also contribute to acyl-CoA–dependent acylation of lysophospholipids and thus generate a phospholipid pool required for efficient sporulation of yeast cells.

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