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Janus-faced Enzymes Yeast Tgl3p and Tgl5p Catalyze Lipase and Acyltransferase 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 GXSXG but also an H-$(X)_4$-D acyltransferase 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 Ale1p 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 Ale1p/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 LPAT, 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-$(X)_4$-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:dihydroxyacetone-phosphate acyltransferase and 2-acylglycerophosphati-

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Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DAG, diacylglycerol; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; PLD, phospholipase D; LPE, lysophosphatidylethanolamine acyltransferase; LPEAT, lysophosphatidylethanolamine acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

Table 1. Strains used in this study

Strain	Genotype	Source
BY4741 wild-type	*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf collection (Frankfurt, Germany)
tgl3Δ tgl4Δ	*his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YMR313c::kanMX4; YKR089c::kanMX4*	Athenstaedt and Daum (2005)
FY1679	*MATa, ura3-52, trp163, leu21, his3200*	Euroscarf collection
TGL3-His_6	plasmid pFA6a-TRP-PGAL1 for N-terminal tagging	This study
BY4743 2n	*MATa/MATa; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0*	Euroscarf collection
tgl3 2n	*BY4743; MAT a/alpha; his3Δ/his3Δ; leu2Δ0/ leu2lys2Δ0/ LYS2; MET15/met15Δ0; ura3Δ0/uraΔ0; YMR313c::kanMX4/YMR313c::kanMX4/::kanMX4*	Euroscarf collection
GS115	*his4*	Invitrogen
Tgl5p-His_6	GS115, TGL5-His_6 tag at the N terminus	This study
tgl3Δ	*MATa, ura3-52, trp1Δ63, leu2Δ1, his3Δ200, Δtgl3::kanMX4*	Euroscarf collection

dylethanolamine acyltransferase (West *et al*., 1997; Beigneux *et al*., 2006). One enzyme of the GPAT family, LPCAT1 from mammalian cells, was cloned and characterized (Chen *et al*., 2006). LPCAT1 specifically prefers saturated fatty acids as acyl donors and is exclusively present in lung alveolar type II cells. Recently Gosh *et al*. reported that *ICT1* (Ghosh *et al*., 2008), *CGI58* (Ghosh *et al*., 2008), and *At4g24160* (Ghosh *et al*., 2009) from yeast, mammalian, and plant systems share the highly conserved motif, containing invariant histidine and aspartic acid residues (H-$(X)_4$-D) and catalysis LPAAT activity.

PA plays an important role in many cellular events such as exocytosis, endocytosis, and signaling through the activation of lipid kinases and protein phosphatases (Sergeant *et al*., 2001; Coon *et al*., 2003). The level of LPAAT is increased in human cancer cells and plays a major role in apoptosis or necrosis (Coon *et al*., 2003). In plants, PA acts on many signaling pathways and its cellular level is increased upon various stress conditions via PLD activation or through DAG kinase (Testerink and Munnik, 2005). Also in the yeast, the level of membrane phospholipids is elevated upon various cellular stress conditions. During sporulation of *Saccharomyces cerevisiae*, an elevated amount of PA produced by the action of Pld1p plays a pivotal role in membrane trafficking (Sreenivas *et al*., 1998; Rudge *et al*., 2004). In addition, PC and PE synthesis are also essential for efficient sporulation, which requires rapid membrane synthesis for prospore membrane formation around each haploid nucleus (Deng *et al*., 2008). In yeast, among the numerous genes involved in spore formation some are specifically required for phospholipid metabolism and related signaling processes (Sarkar *et al*., 2002; Neiman, 2005; Li *et al*., 2007).

Previous studies from our group showed that the yeast TAG hydrolytic enzymes Tgl3p and Tgl5p seemed to be involved in spore formation (Athenstaedt and Daum, 2003; 2005). This finding and the above-mentioned evidence for the role of phospholipids in yeast sporulation led us to investigate a possible metabolic link between TAG hydrolysis and phospholipid synthesis, in particular acyltransferase reactions that might be involved in the regeneration of membrane lipids. In the course of the biochemical characterization of Tgl3p and Tgl5p, we found that both polypeptides do not only contain the lipase characteristic amino acid sequence GXSXG, but also an acyltransferase signature motif H-$(X)_4$-D. Data presented here demonstrate that the overexpression of these two TAG lipases distinctly enhanced the level of phospholipids, confirming the possible additional role of these enzymes as acyltransferases. Indeed, purified Tgl3p and Tgl5p showed acyl-CoA dependent acylation of LPE and LPA, respectively. So far, it has been reported that putative lipases and phospholipases can mediate acyl-CoA independent acylation or transacylation reaction thereby participating in membrane lipid remodeling (Ma and Turk, 2001; Jenkins and Frohman, 2005; Pérez *et al*., 2004). Our results show for the first time that the yeast TAG lipases Tgl3p and Tgl5p play a dual role in lipid metabolism and function in both anabolic and catabolic pathways of *S. cerevisiae*.

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_6-tagged Tgl5p, *Pichia pastoris* cells were grown on buffered minimal methanol medium (BMM10) containing 1.34% yeast nitrogen base, 4×10^{-4} % biotin (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 23°C.

Bioinformatics Analysis

Conserved protein domains and motifs were examined using the CDD at National Center for Biotechnology Information (http://www.ncbi.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 Axiovert 35 microscope (Carl Zeiss, Jena, Germany), with a 100-fold oil immersion objective.

Site-directed Mutagenesis

Plasmid pYES2TGL3^{wt} was obtained as described previously by Athenstaedt and Daum (2003). Plasmids pYES2TGL3^{S237A}, pYES2TGL3^{H298A}, pYES2TGL3^{D303A}, and pYES2TGL3^{D303E} were constructed using the QuikChange polymerase chain reaction (PCR)-based mutagenesis procedure (Stratagene, La Jolla, CA) with the pYES2TGL3^{wt} 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_6-tagged hybrids of Tgl3p under a galactose-inducible promotor in *S. cerevisiae* has been described by Athenstaedt and Daum (2003).

Table 2. Oligonucleotides for site-directed mutagenesis of *TGL3*

Mutation	Oligonucleotide sequence
S237A	(+) 5′-CTAATATTATAAGTGGTAGCGCCATGGGGGCATGCGTTG-3′
	(−) 5′-CAACGCATGCCCCCATGGCGCTACCACTTATAATATTAG-3′
H298A	(+) 5′-CTTTGATTCAGAACCTAATACACGCCGGTTATTCTCAAGACGTTTATC-3′
	(−) 5′-GATAAACGTCTTGAGAATAACCGGCGTGTATTAGGTTCTGAATCAAAG-3′
D303A	(+) 5′-CACCACGGTTATTCTCAAGCCGTTTATCTTTTTATCCGG-3′
	(−) 5′-CCTTCTCGGAAGAGCCGGGTACTGGAGGCCTCATATGTGAGG-3′
D303E	(+) 5′-CACCACGGTTATTCTCAAGAGGTTTATCTTTTTATCCGG-3′
	(−) 5′-CCGGATAAAAAGATAAACCTCTTGAGAATAACCGTGGTG-3′

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

For the isolation of the His-tagged Tgl3p, 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 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) for 30 min on ice. The sample was centrifuged for 15 min at 13,000 × *g* at 4°C, and solubilized proteins were separated from the floating lipid layer. Solubilized lipid particle proteins were immediately applied onto a 1-ml His-Trap chelating column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using the standard protocol of the manufacturer. The column was washed with 10 ml of equilibrium buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 20 mM imidazole, and 50 μM 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 12.5 μM CHAPS). Fractions of 1 ml were collected and aliquots of 90 μl were used for measuring the acyltransferase activity as described below.

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, 0.3 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). For Western blot analysis, 1 μg of total protein was separated by 12% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare) by standard procedures described previously (Haid and Suissa, 1983). Immunoreactive protein bands were detected by enzyme-linked immunosorbent assay using rabbit or mouse antisera as the first antibody and goat anti-rabbit or goat anti-mouse IgG, respectively, linked to peroxidase as the second antibody.

Metabolic Labeling of Phospholipids and Neutral Lipids

The Tgl3p-His_6–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 [^{14}C]acetate (specific activity 51 mmol/mCi) and grown for additional 24 h. Cells (OD 10) were harvested by centrifugation, and lipids were extracted using chloroform/methanol (2:1; vol/vol). Individual phospholipids were separated by two-dimensional thin layer chromatography (TLC) on silica gel 60 using chloroform/methanol/25% ammonia (65:35:5, per vol) as a first developing solvent, and chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, per vol) as a solvent for the second dimension. Neutral lipids were separated by one-dimensional TLC by using light petroleum:diethyl ether:acetic acid (70:30:1, per vol) as the solvent system. The wild type *Pichia pastoris* GS115 strain and GS115 containing the Tgl5p-His_6 hybrid were grown in BMM10 medium with 1 μCi/ml [^{14}C]acetate for the in vivo labeling studies as described above.

In Vitro Lysophospholipid Acyltransferase Assay

Lysophospholipid acyltransferase activity was determined by measuring the incorporation of [^{14}C]oleoyl-CoA into phospholipids in the presence of lysophospholipids as acyl acceptor molecules. Lysophospholipids used were 1-oleoyl-sn-glycero-3-phosphocholine, 1-oleoyl-*sn*-glycerol-3-phosphate (sodium salt), 1-acyl-*sn*-glycero-3-phospho(2-aminoethanol) from egg yolk, 1-acyl-*sn*-glycero-3-phospho-(1-D-myo-inositol) from *Glycine max* and 1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phospho-L-serine (sodium salt). Lysolipids were purchased from Sigma-Aldrich and Avanti Polar Lipids (Alabaster, AL). All reactions were performed in the presence of 100 mM Tris-HCl, pH 7.5, 100 μM of the respective lysophospholipids, and 20 μM [^{14}C]oleoyl-CoA (58 mCi/mmol) at 30°C with 0.2–1 μg of purified TAG lipase proteins Tgl3p or Tgl5p in a total volume of 100 μl for 15 min. The reaction was stopped by extracting lipids with 800 μl of chloroform/methanol/2% phosphoric acid (1:2:1, per vol). Subsequently, lipids were separated by one-dimensional TLC by using chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, per vol) as the solvent system. Individual lipid spots 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 (Mallinckrodt 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 dithiothreitol. The substrate mixture containing 20 μM egg phospholipids (PC) and 100 μM [9,10-^{3}H]triolein were 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 (Athenstaedt 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 *tgl3*Δ deletion and *TGL3* overexpressing strains. For the latter purpose, we used a strain that overexpressed a Tgl3p-His_6 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-

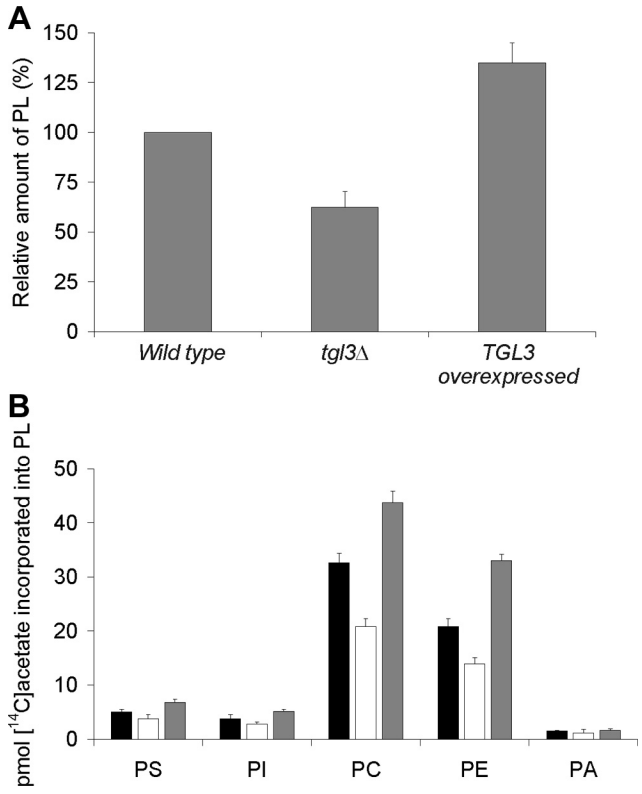

Figure 1. Overexpression of *TGL3* increases the amount of phospholipids. (A) Amount of total phospholipids per mg protein from whole cell extracts was quantified as described under *Materials and Methods*, and the value of wild type was set at 100%. (B) Amount of [^{14}C]acetate incorporated into individual phospholipids is shown. Data are expressed as picomoles of [^{14}C]acetate incorporated per A_{600} units. Black bars, wild type; white bars, tgl3Δ; and gray bars, Tgl3p overexpressing strain. Mean values of three independent experiments ± SD are shown.

lenged the "traditional" enzymatic properties of Tgl3p as a TAG lipase and tempted us to speculate about possible additional enzyme activities of this protein.

In Silico Analysis of Tgl3p

Tgl3p has been reported to be a member of α/β-hydrolase family of proteins. The pfam analysis and specific motif search revealed that Tgl3p possesses a core patatin domain (PF01734) containing the highly conserved signature motif ^{235}GXSXG^{239} (Figure 2). This motif seems to be critical for hydrolytic activities and conserved among all known

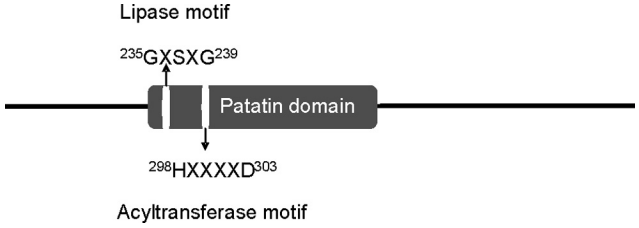

Figure 2. Dual signature motifs of Tgl3p. The patatin domain (P40308) structure of *TGL3* as determined by pfam analysis is indicated by the shaded region. The ^{298}HXXXXD^{303} is a signature motif for all glycerolipid acyltransferases; and ^{235}GXSXG^{239} is a typical lipase motif.

lipases, phospholipases, lysophospholipases, esterases and serine proteases (Teter *et al*., 2001). Surprisingly, we also noticed that Tgl3p contains another distinct motif, namely, ^{298}HXXXXD^{303} (PF00698) at the patatin region (Figure 2). This sequence has been reported as one of the well conserved acyltransferase motifs in *Escherichia coli*, yeast, plants, and mouse (Heath and Rock, 1998; Dircks *et al*., 1999; Lewin *et al*., 1999; Ghosh *et al*., 2008). It was suggested that motif I (H-(X)$_4$-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.

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 His$_6$-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 [^{14}C]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 [^{3}H]dihydrosphingosine 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 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 [^{3}H]triacylglycerol or [^{3}H]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 Zn^{2+} ion but insensitive to Mg^{2+}, whereas LPAAT (Slc1p) activity was enhanced in the presence of Mg^{2+} (Benghezal *et al*., 2007; Chen *et al*., 2007). We found that addition of Cu^{2+} (0.25 mM) and Zn^{2+} (0.5 mM) to the assay mixture led to a 50% inhibition of acyltransferase activity of Tgl3p and that the presence of 1 mM Cu^{2+} and 2 mM Zn^{2+} inhibited the enzymatic activity completely. Other divalent cations such as Ca^{2+}, Mn^{2+} and Mg^{2+} had no effect (data not shown). These data also gave insight that LPEAT activity mea-

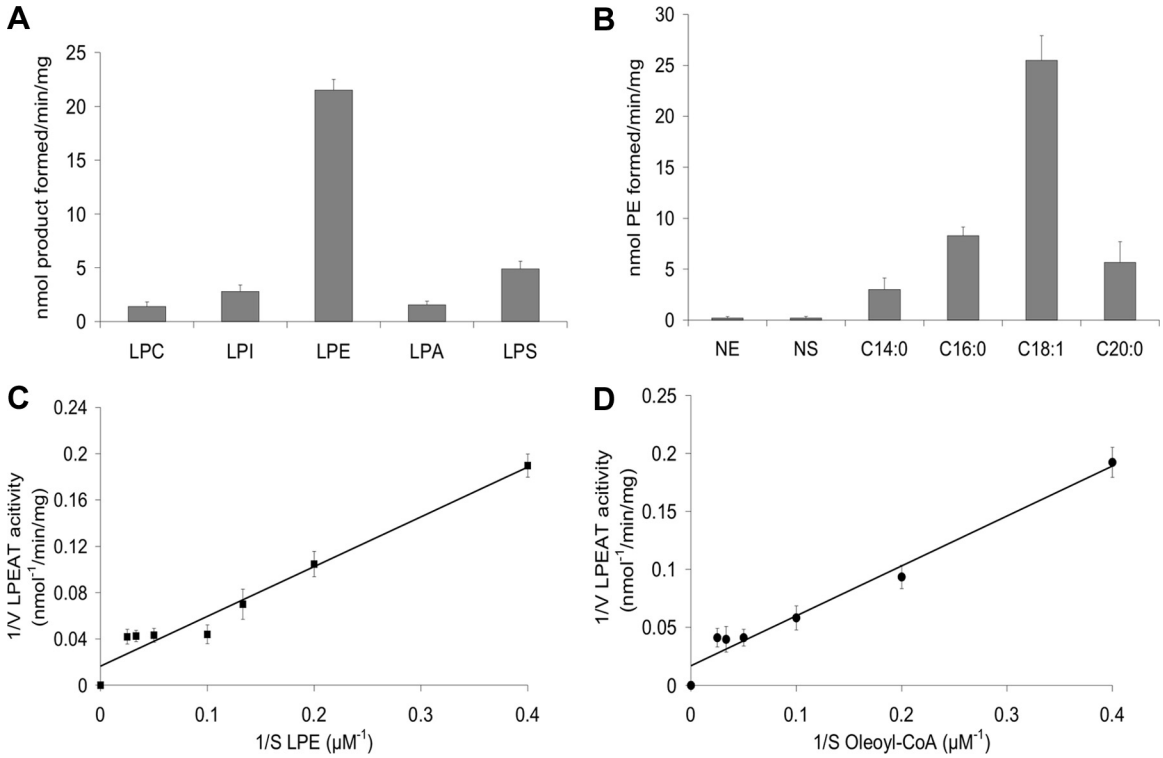

Figure 3. Tgl3p mediates oleoyl-CoA dependent acylation of lysophosphatidylethanolamine. A Tgl3p-His_6 fusion protein was used to analyze enzyme activities. Acyltransferase assays were performed as described in *Materials and Methods*. (A) Purified Tgl3p was examined for the acyl acceptor specificity 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.

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-(X_4)-D (^{298}HGYSQD^{303}). 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-(X_4)-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

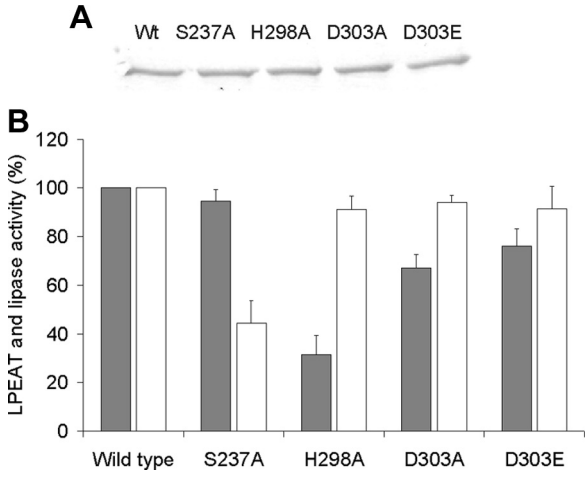

Figure 4. Effect of amino acid substitution on lipase and acyltransferase activity of Tgl3p. Amino acid residues of the acyltransferase motif HGYSQD were replaced by site-directed mutagenesis as indicated. Mutagenized variants were overexpressed using a galactose inducible promoter, lipid particles were 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.

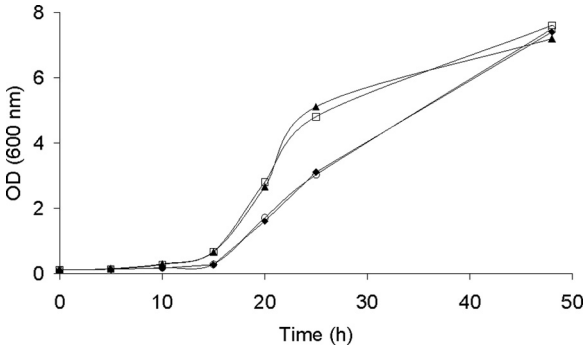

Figure 5. Role of Tgl3p on cell growth. Deletion mutant *tgl3*Δ *tgl4*Δ was transformed with empty pYES2 vector, pYES2-*TGL3*^{wt}, pYES2-TGL3^{S237A}, and pYES2-*TGL3*^{H298}. All transformants were pre-cultured in minimal glucose medium without uracil at 30°C for 48 h to reach the stationary phase. Then, cells were inoculated into fresh induction medium containing galactose as the carbon source for growth phenotype analysis. *Open circles: tgl4*Δ/pYES2^{vector}; Open squares, *tgl3*Δ *tgl4*Δ/pYES2-*TGL3*^{wt}; filled triangles, *tgl3*Δ *tgl4*Δ/pYES2-TGL3^{S237A}; and filled diamonds, *tgl3*Δ *tgl4*Δ/pYES2-*TGL3*^{H298A}.

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 *tgl3*Δ*tgl4*Δ 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 *tgl3*Δ *tgl4*Δ 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-Tgl3p*^{Wt} and *pYES2-Tgl3p*^{S237A} rescued the delayed growth of *tgl3*Δ *tgl4*Δ. 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 *tgl3*Δ *tgl4*Δ 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 (Primig *et al*., 2000). As mentioned above, the Tgl3p and Tgl5p are maximally expressed during sporulation (www.yeastgenome.org), and homozygous *tgl3*Δ/*tgl3*Δ and *tgl4*Δ*tgl5*Δ/*tgl4*Δ*tgl5*Δ deletion mutants are defective in sporulation. This information led us to elucidate an apparent role of this gene during yeast spore formation and to investigate whether lipase or acyltransferase functions of Tgl3p play a key role in this process. Hence, the homozygous diploid *tgl3*Δ/*tgl3*Δ mutant was transformed with empty pYES2 plasmid (vector control), pYES2-*TGL3*^{WT},

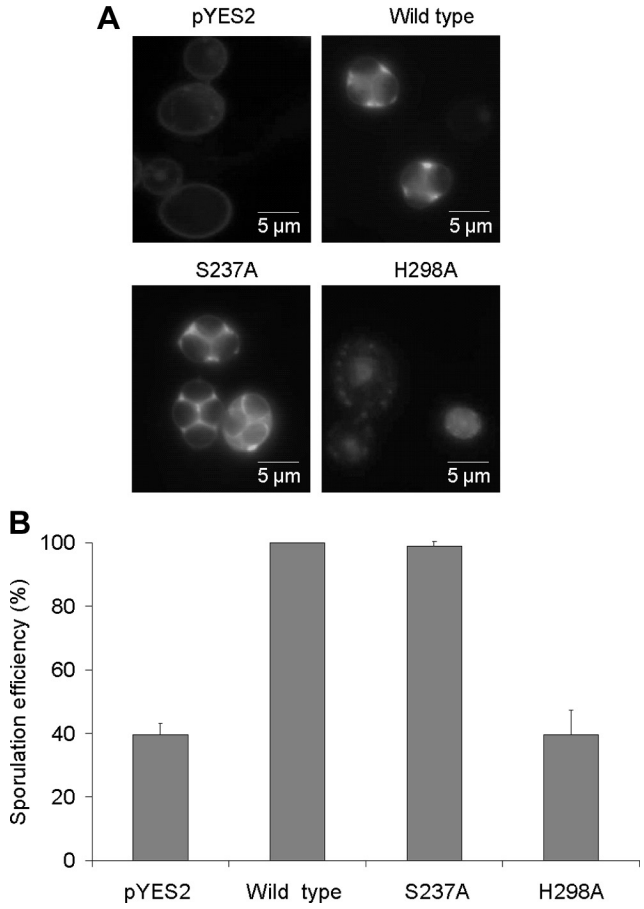

Figure 6. Functional role of Tgl3p during yeast spore formation. A *tgl3*Δ *tgl3*Δ homozygous diploid and *tgl3*Δ *tgl3*Δ transformed with plasmids bearing wild-type *TGL3* and variants, respectively, were grown in sporulation medium as described in *Materials and Methods*. (A) DAPI staining. (B) Spores in cultures were counted and sporulation efficiency was calculated as percentages. Mean values of three independent experiments ± SD are shown.

pYES2-*TGL3*^{S237A} and pYES2-*TGL3*^{H298A} and the efficiency of spore formation was tested (Figure 6A). We observed that the *tgl3*Δ/*tgl3*Δ mutant carrying pYES2-*TGL3*^{WT} and *TGL3* bearing a mutation in the lipase motif (pYES2-*TGL3*^{S237A}) sporulated as efficiently as the wild-type diploid strain, whereas the *TGL3* variant with the point mutation in the acyltransferase motif (pYES2-*TGL3*^{H298A}) 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 Lysophosphatidic 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 with GXSXG (^{214}GSSAG^{218}) as a consensus sequence for lipases. This domain, however, also contains the typical motif H-$(X)_4$-D (^{54}HAISYD^{59}), which is crucial for acyltransferase activity. Thus, sequence motifs of Tgl5p resemble those of Tgl3p (Figure 2). In contrast, Tgl4p does not

have an H-$(X)_4$-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}C]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_6-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 ± 1.3 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 oleoylCoA as substrates (Figure 8, B and C). We further analyzed the acyl chain selectivity of Tgl5p by using [1-^{14}C]palmitoyl-CoA (16:0) or [1-^{14}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).

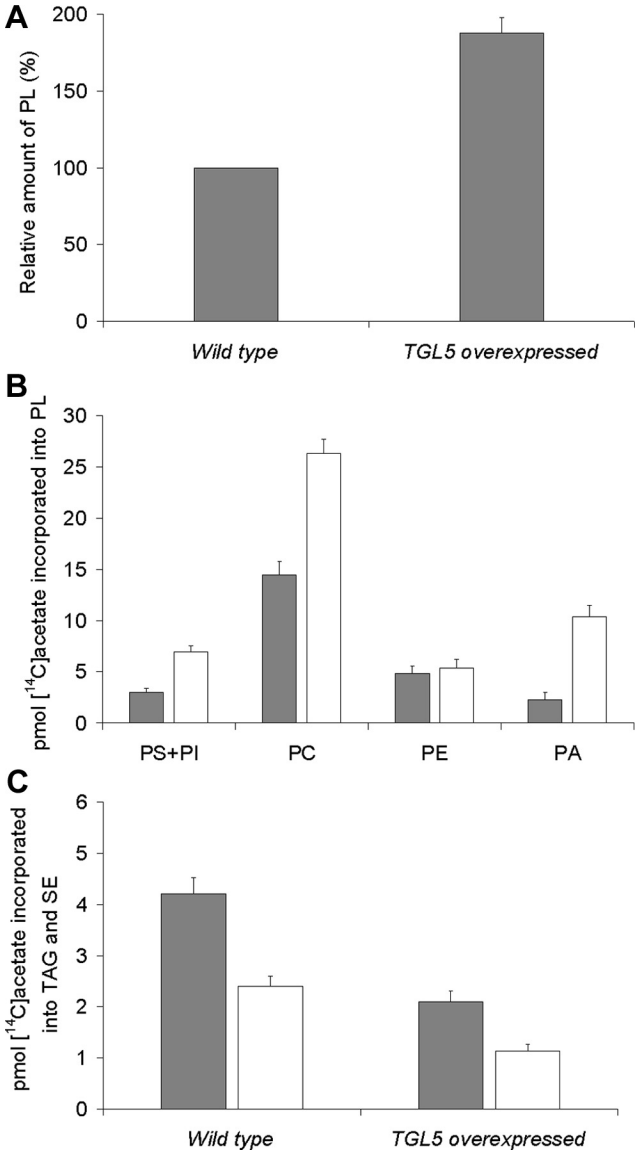

Figure 7. Heterologous overexpression of Tgl5p in *P. pastoris* enhances synthesis of phosphatidic acid. (A) Amount of total phospholipids per milligram of protein from whole cell extracts was quantified as described under *Materials and Methods*, and the value of wild type was set at 100%. (B) Cells overexpressing Tgl5p and the wild type were labeled in the presence of [^{14}C]acetate. Incorporation of [^{14}C]acetate into individual phospholipids was measured and used to calculate phospholipid synthesis. Black bars, wild type cells; and white bars, Tgl5p-overexpressing strain. (C) Incorporation of [^{14}C]acetate into neutral lipids. Black bars, TAG; and white bars, SE. Mean values of three independent experiments ± SD are 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 *tgl3*Δ/*tgl3*Δ 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 ability of Tgl3p and Tgl5p to act as lysophospholipid acyltransferases 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 acyltransferases, and an *slc1*Δ *slc4*Δ 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

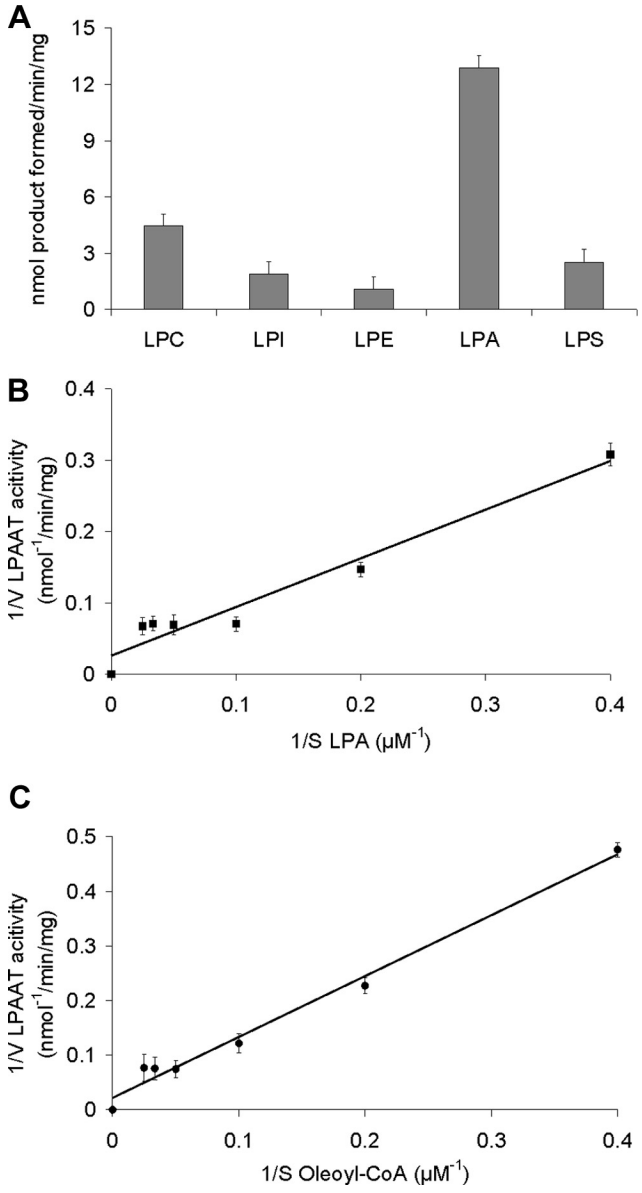

Figure 8. Tgl5p catalyzes oleoyl-CoA–dependent acylation of lysophosphatidic acid. A purified Tgl5p-His_6 fusion protein was examined for lysophospholipid acyltransferase activity. (A) Examination of different lysophospholipid acceptors in the presence of 18:1 acyl-CoA. (B) Lineweaver-Burk plot for variable amounts of LPA used as substrate. (C) Lineweaver-Burk plot for variable amounts of oleoyl-CoA used as substrate. The mean values of three independent experiments ± SD are shown.

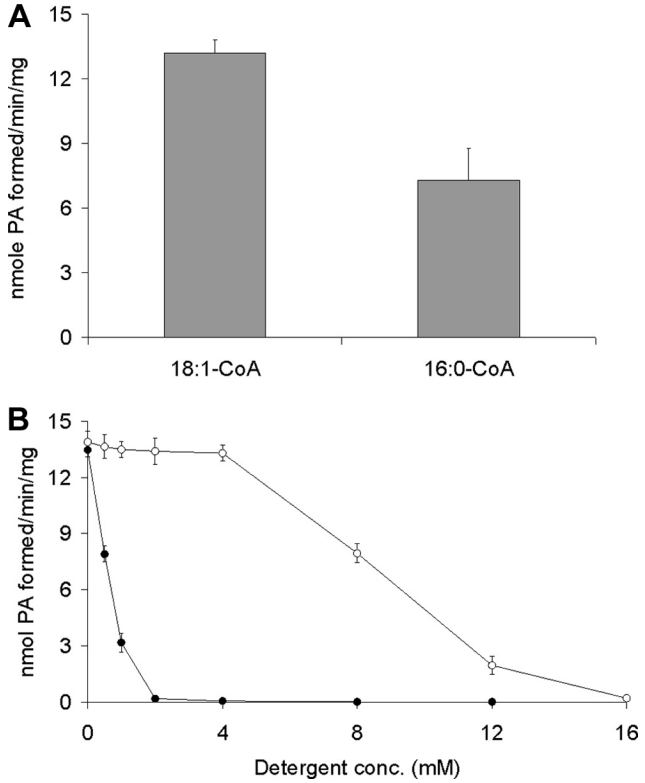

Figure 9. Characterization of the purified Tgl5p. (A) Acyl chain preference of purified Tgl5p. (B) Effect of detergents on LPA acyltransferase activity. Open circles, CHAPS; and filled circles, Triton X-100.

less all lysophospholipid. Therefore, it was not surprising that overexpression of Tgl3p in the *slc1*Δ *slc4*Δ double mutant background did not rescue the synthetic lethal phenotype (data not shown). Moreover, Slc1p has Mg^{2+}-dependent LPAAT activity, whereas purified Tgl3p was Mg^{2+} independent in vitro and Cu^{++} and Zn^{++} 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 phospholipid 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 stationary phase to the lag phase. Our results presented here suggest 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 *tgl3*Δ *tgl4*Δ double deletion strain (Figure 5B) was specifically rescued by overexpression of the $Tgl3p^{S237A}$ variant (acyltransferase active) but not by $Tgl3p^{H298A}$ (acyltransferase inactive). This result illustrated that the acyltransferase but not the TAG lipase function of Tgl3p is essential for membrane lipid biosynthesis during the lag phase.

During sporulation, the meiotic progression is accompanied by the formation of a prospore membrane which requires membrane lipid synthesis. In general, phospholipases and lipases play critical roles in cell signaling by producing second messenger lipids such as DAG, PA, and lysophospholipids. 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 (Honigberg *et al*., 1992; Rose *et al*., 1995; Rudge *et al*., 2004). Expression 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 observation that homologous diploid strains of *tgl3*Δ/*tgl3*Δ and *tgl4*Δ *tgl5*Δ/*tgl4*Δ *tgl5*Δ double deletion strains bear a sporulation defect (Athenstaedt and Daum, 2005). Our analysis toward the dual functions of TAG lipases upon yeast sporulation 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 formation and maintaining the structural integrity of prospore 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 hydrolases, 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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