Regulation of the Yeast Triacylglycerol Lipase Tgl3p by Formation of Nonpolar Lipids*

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Background: Tgl3p from yeast serves as the major triacylglycerol lipase but also as lysophospholipid acyltransferase.

Results: Formation of nonpolar lipids strongly affects subcellular localization and function of Tgl3p.

Conclusion: Tgl3p activity is mainly regulated by the presence/absence of lipid droplets.

Significance: The yeast lipase/acyltransferase Tgl3p is an important player in lipid homeostasis.

Tgl3p, the major triacylglycerol lipase of the yeast *Saccharomyces cerevisiae*, is a component of lipid droplets but is also present in the endoplasmic reticulum in a minor amount. Recently, it was shown that this enzyme can also serve as a lysophospholipid acyltransferase (Rajakumari, S., and Daum, G. (2010) Mol. Biol. Cell 21, 501–510). Here, we describe the effects of the presence/absence of triacylglycerol and lipid droplets on the functionality of Tgl3p. In a *dga1Δ/are1Δ/are2Δ* quadruple mutant lacking all four triacylglycerol- and sterol ester-synthesizing acyltransferases and consequently the lipid droplets, the gene expression of *TGL3* was only slightly altered. In contrast, protein level and stability of Tgl3p were markedly reduced in the absence of lipid droplets. Under these conditions, the enzyme was localized to the endoplasmic reticulum. Even the lack of the substrate, triacylglycerol, affected stability and localization of Tgl3p to some extent. Interestingly, Tgl3p present in the endoplasmic reticulum seems to lack lipolytic as well as acyltransferase activity as shown by enzymatic analysis and lipid profiling. Thus, we propose that the activity of Tgl3p is restricted to lipid droplets, whereas the endoplasmic reticulum may serve as a parking lot for this enzyme.

All types of eukaryotes, including the yeast *Saccharomyces cerevisiae*, contain lipid droplets (LD)† (1–3). These organelles, also known as lipid particles or oil bodies, serve as a storage compartment for nonpolar lipids. In *S. cerevisiae*, triacylglycerols (TG) and sterol esters (SE) are the two major classes of nonpolar lipids. These components provide a source of energy, but they also serve as important depots of building blocks for the formation of membrane phospholipids. The structure of LD is largely conserved in all eukaryotes (4). In the yeast, LD contain a hydrophobic core of TG and SE, ~50% each, which is surrounded by a phospholipid monolayer with a small but distinct set of proteins embedded (4–6).

The biogenesis of LD is still a matter of dispute. It is generally accepted that LD are derived from the endoplasmic reticulum (ER) where the majority of nonpolar lipids are synthesized (7, 8). In various models that describe the formation of LD (for a recent review see Ref. 9), TG and SE accumulate in the ER, are enwrapped by a phospholipid monolayer and, leave the ER when reaching a critical size. LD are dynamic organelles and remain functionally and physically connected to the ER (10).

During the last few years, many enzymes involved in the formation of nonpolar lipids have been identified and characterized. In the yeast *S. cerevisiae*, four acyltransferases contribute to nonpolar lipid synthesis, namely Lro1p, Dga1p, Are1p, and Are2p (11). At an early growth stage, TG are mainly formed by the phospholipid:diacylglycerol acyltransferase Lro1p, which is exclusively localized to the ER (12, 13). The acyl-CoA independent enzyme requires a phospholipid, preferentially phosphatidylethanolamine or phosphatidylcholine, as an acyl donor and resembles the human lecithin:cholesterol acyltransferase. Alternatively, TG is formed by the acyl-CoA:diacylglycerol acyltransferase Dga1p, which is dually located to LD and the ER (14, 15). Dga1p uses activated fatty acids as a co-substrate and is the major TG synthase in *S. cerevisiae* grown under standard conditions, especially when cells reach the stationary phase (16). SE of *S. cerevisiae* are synthesized by two acyl-CoA:sterol acyltransferases named Are1p and Are2p (17), two closely related enzymes located to the ER. Are1p and Are2p exhibit slight differences in their substrate specificities (18). The major SE synthase Are2p preferentially utilizes ergosterol as a substrate, whereas Are1p esterifies ergosterol precursors, mainly lanosterol, as well. Additionally, the two SE synthases contribute to TG synthesis although with minor efficiency (16). A yeast strain lacking all four nonpolar lipid-synthesizing enzymes, the *dga1Δ/are1Δ/are2Δ* quadruple mutant (QM), is still viable under standard growth conditions but does not form LD (16). In this mutant, several LD proteins are retained in the ER (19).

Mobilization of TG from LD requires hydrolytic enzymes located at the surface monolayer membrane of LD (20–22). The three yeast TG lipases identified so far, Tgl3p, Tgl4p, and Tgl5p, catalyze hydrolysis of TG to diacylglycerols (DG) and free fatty acids. Interestingly, previous studies from our labora-

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‡ The abbreviations used are: LD, lipid droplet; DG, diacylglycerol; ER, endoplasmic reticulum; QM, quadruple mutant; SE, sterol ester; TG, triacylglycerol.
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Experimental Procedures

Strains and Culture Conditions—Yeast strains used in this study are listed in Table 1. Cells were grown aerobically either to the logarithmic or to the stationary growth phase at 30 °C in YPD media containing 1% yeast extract, 2% glucose, and 2% peptone. Yeast strains bearing plasmids were cultivated in synthetic minimal medium containing 0.67% yeast nitrogen base (U. S. Biochemical Corp.), 2% glucose, and the respective amino acid supplements. Growth was monitored by measuring absorbance at 600 nm (A600) respectively, as substrates (23–25). In vivo experiments indicated with trichloroacetic acid at a final concentration of 10%.

Isolation and Characterization of Subcellular Fractions—Highly purified LD and microsomes were isolated from cells grown to the stationary phase following published procedures (4, 28, 29). The protein concentration of isolated fractions was analyzed by the method of Lowry et al. (30) using bovine serum albumin as a standard. Prior to protein analysis, samples of LD fractions were delipidated with 2–3 volumes of diethyl ether. The organic phase was withdrawn, and residual diethyl ether was removed under a stream of nitrogen. Proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 100 μl of 0.1% SDS, 0.1 M NaOH.

SDS-PAGE was carried out by the method of Laemmli (31) using 12.5% separation gels, and Western blot analysis was performed as described by Haid and Suissa (32). Proteins were detected by using rabbit or mouse antisera as the first antibody and peroxidase-conjugated goat anti-rabbit or anti-mouse IgG.
as the second antibody. Primary antibodies were directed against the Myc tag/HA tag. Wbp1p (ER marker), GAPDH (cytosolic marker), and Ayr1p, Erg6, and Erg1p (LD markers). 10 μg of each fraction were loaded onto SDS gels for Western blot analysis. Comparative immunoblot data were from the same blot. Relative intensities of Western blots were calculated using the ImageJ program.

Preparation of Total Cell Extract for Lipid Analysis—Total cell extract for lipid analyses was prepared by growing yeast cells to the stationary phase. Cells were harvested by centrifugation at 3,000 × g for 5 min at room temperature. The cell pellet was resuspended in breaking buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl) with addition of PMSF (1 mM). Cells were disintegrated by vigorous shaking in the presence of glass beads for 10 min at 4 °C. After disruption, cell debris was removed by centrifugation at 3,000 × g for 5 min. The supernatant was further used for protein determination and lipid extraction.

Lipid Analysis—Lipids from total cells were extracted as described by Folch et al. (33) using chloroform/methanol (2:1; v/v) as solvent. For quantification of DG, a lipid extract of total cell extracts (200 μg of protein) was separated by thin layer chromatography (TLC) using Silica Gel 60 plates (Merck). Chromatograms were developed in an ascending manner using chloroform/acetone/acidic acid (45:4:0.5 per volume) as a solvent system. Bands were visualized by dipping the plate for 10 s into a solution consisting of 0.63 g of MnCl₂·4H₂O, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulfuric acid and incubated in a heating chamber at 105 °C for at least 30 min. The DG bands were then quantified by densitometric scanning at 400 nm with a TLC scanner (CAMAG TLC Scanner 3). Diolein served as a standard.

For quantification of total phospholipids, a lipid extract of total cell extracts (800 μg of protein) was analyzed by the method of Broekhuysen (34). Individual phospholipids were analyzed from total cell lipid extracts (2 mg of protein) by two-dimensional TLC using chloroform/methanol/25% ammonia (65:35:5 per volume) as solvent system for the first dimension, and chloroform/acetone/methanol/acidic acid/water (50:20:10:10:5 per volume) for the second dimension. Spots were visualized by staining with iodine vapor, scraped off, and quantified by the method of Broekhuysen (34).

Enzyme Assays—TG lipase activity of isolated subcellular fractions was determined using LD (5–10 μg of protein) or the 30,000 × g ER fraction (300 – 400 μg of protein) as an enzyme source. Lipase activity was measured in a final volume of 200 μl. Samples were incubated in a mixture containing 100 mM potassium phosphate buffer, pH 7.5, containing 250 μM [9,10-3H]triolein (specific activity of 33 μCi/ml), 45 μM phosphatidylcholine/phosphatidylinositol (3:1; mol/mol), 25 μM MgCl₂, and 0.2% fatty acid-free BSA at 30 °C for 1 h in a water bath. The substrate was prepared as follows. Triolein and phosphatidylcholine/phosphatidylinositol were dried under a stream of nitrogen and emulsified by sonication for 4 min at 30 °C in potassium phosphate buffer with addition of BSA.

The reaction was stopped by adding 3 ml of chloroform/methanol (2:1, v/v), and lipids were extracted by vortexing. The lipid extract was dried under a stream of nitrogen and then dissolved in 40 μl of chloroform/methanol (2:1, v/v) and separated by TLC. Chromatograms were developed in an ascending manner using chloroform/acetone/acidic acid (45:4:0.5 per volume) as a solvent system. Fatty acid bands were scraped off the plate, and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as scintillation mixture.

Lysophospholipid acyltransferase activity of isolated subcellular fractions was measured using 20 μg of protein of 30,000 × g ER as an enzyme source in a final volume of 200 μl. The assay mixture contained 100 mM Tris·HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 100 μM lysophosphatidylethanolamine from egg yolk (Sigma) and 20 μM [14C]oleoyl-CoA (41.9 mCi/mmol). Samples were incubated at 30 °C for 2 min in a water bath. The reaction was stopped as described above. Chromatograms were developed in an ascending manner using chloroform/acetone/methanol/acidic acid/water (50:20:10:10:5 per volume) as a solvent system. Phosphatidylethanolamine bands were scraped off the plate, and radioactivity was measured as described above.

RNA Isolation and Real Time PCR—Total RNA from cells grown to the mid-logarithmic phase on YPD at 30 °C were isolated using an RNeasy kit from Qiagen by following the manufacturer’s instructions. After DNase I digestion, real time PCR was performed using SuperScript III Platinum SYBR Green one-step quantitative RT-PCR kit (Invitrogen) as described by the manufacturer. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the ΔΔCt method described by
Livak and Schmittgen (35). With this method, the differences in mRNA expression after ACTI normalization relative to the control can be calculated. Primers used for real time PCR are listed in Table 3.

Fluorescence Microscopy—*S. cerevisiae* cells were grown in synthetic minimal medium containing glucose to the late logarithmic phase. For the induction of hybrid protein expression, an aliquot of the culture was shifted to galactose-containing medium for 4–6 h. LD were stained with the hydrophobic dye Nile Red (Sigma) (1 μg/ml) for 5–10 min at room temperature. Cells were washed twice with sterile water and further used for fluorescence microscopy. Fluorescence microscopy was carried out on a Zeiss Axioskop microscope using a ×100 oil immersion objective with a narrow band enhanced GFP and dsRed filter set (Zeiss). Images were taken with a Visicam CCD camera and displayed using the Metamorph Imaging software (Visiteron Systems, Puchheim, Germany). Exposure time for visualization was 10 s for ER proteins and 300 ms for LD proteins. Transmission images were obtained by using Nomarski optics (differential interference contrast).

RESULTS

Lack of Nonpolar Lipids Affects Gene Expression, Protein Level, and Stability of Tgl3p—The major TG lipase Tgl3p from *S. cerevisiae* is a component of LD where it plays a critical role in TG mobilization. Although molecular functions of the enzyme were studied in some detail by Rajakumari and Daum (25), evidence about the mechanisms regulating the activity of Tgl3p is limited.

A major question addressed in this study was how Tgl3p behaves in the absence of nonpolar lipids and consequently in the absence of LD. We first examined the expression level of TGL3 under these conditions. As can be seen from Fig. 1A, gene expression of *TGL3* was only slightly reduced in a *dga1Δ mro1Δare1Δare2Δ* QM, which is devoid of LD. This finding was surprising, because the lipase substrate TG is missing in this strain. Interestingly, however, the protein level of Tgl3p in this mutant was markedly lower than in wild type (Fig. 1B). Quantitative Western blot analysis revealed a reduction of the total amount of Tgl3p to 50% of wild type (Fig. 1C). The strongly reduced amount of Tgl3p despite the largely unaffected transcription of *TGL3* tempted us to speculate that decreased protein stability might be the reason for the reduced steady state level of the protein. To test this hypothesis, wild type and QM were grown to the mid-logarithmic phase and poisoned with cycloheximide. The degradation of Tgl3p in the absence of protein synthesis was monitored over 24 h. Western blot analysis clearly revealed that the stability of Tgl3p was strongly reduced in the absence of LD (Fig. 1D). The protein half-life of Tgl3p in the QM was 3 h, whereas that of wild type Tgl3p was ~24 h (Fig. 1E).

Localization and Lipolytic Activity of Tgl3p in the Absence of LD—In wild type cells, the majority of Tgl3p is located to the surface phospholipid monolayer of LD (Fig. 2A). The presence of substantial amounts of Tgl3p in the QM raised the question about the subcellular distribution of the protein in this strain. Previously, it was reported that some LD proteins were retained in the ER of yeast cells lacking nonpolar lipids and hence LD (19) reflecting the close relationship of the two organelles. Therefore, we speculated that such a subcellular rearrangement might also occur with Tgl3p. To test this hypothesis, we isolated microsomes from QM and the corresponding wild type strain and tested for the presence of Tgl3p. In wild type, the Myc-tagged Tgl3p was localized to LD at high abundance, confirming that the tag did not influence the subcellular distribution of the protein (Fig. 2A). Also, other tags did not influence localization and functionality of the enzyme. However, smaller amounts of Tgl3-Myc were also detected in 30,000 × g (M30) and 40,000 × g microsomes (M40) from wild type cells. This result is in contrast to previous studies (20) where a less sensitive assay system did not detect Tgl3p in microsomes. Thus, results presented here demonstrate that Tgl3p also belongs to the group of proteins that are dually located to LD and the ER. The minor signal of Tgl3-Myc in the cytosolic fraction of wild type is most likely due to residual LD. Most importantly, the amounts of Tgl3p present in the homogenate and in microsomes from the QM showed almost the same enrichment pattern as the ER marker Wbp1p. Thus, in cells lacking LD, Tgl3p seems to behave like a "true" ER protein.

Fluorescence microscopy confirmed the results obtained by Western blot analysis. For this purpose, we used GFP fused to the N terminus of Tgl3p under a galactose-inducible promoter. In the wild type background, GFP-Tgl3p was located to LD (Fig. 2B) and co-localized with Nile Red confirming previous results from our laboratory (20). In the QM, GFP-Tgl3p was enriched in the nuclear ER exhibiting co-localization with the ER marker protein Sec61-mCherry.

Lipase activity of Tgl3p in the QM does not seem to be relevant in vivo, because the substrate TG is not available. Nevertheless, we tested TG lipolytic activity of Tgl3p when located to the ER. Fig. 2C shows *in vitro* TG activity of LD and ER fractions from wild type and QM overexpressing *TGL3*. LD from wild type exhibited a specific lipolytic activity of 0.19 ± 0.045 pmol of fatty acids formed/h/mg of protein with [9,10-3H]triolein as substrate. However, ER fractions from both wild type and QM showed only marginal TG activity. Thus, it appears that Tgl3p is not an active lipase when located to the ER.

Substrate Availability Affects Regulation of Tgl3p—Experiments described above clearly demonstrated that protein level, stability, and localization of Tgl3p are dramatically altered in yeast cells lacking nonpolar lipids and LD. The question remained whether or not the absence of the major substrate of Tgl3p, TG, is already sufficient to cause the observed effects. Therefore, we examined gene expression, protein level, stabil-

\[3\text{C. Schmidt, K. Athenstaedt, B. Koch, B. Ploier, and G. Daum, unpublished results.}\]

| Primer       | Sequence (5′ → 3′)         |
|--------------|---------------------------|
| RT Act1-Fwd  | CCAGGCTTCTACGTGTCATCCAAG  |
| RT Act1-Rev  | GACGTGAGTAACACCATCACGGA   |
| RT Tgl3-Fwd  | GCCAAACATCGGACGATAACGGAG  |
| RT Tgl3-Rev  | TGTTGCCAACATATGTTCTCGCCA  |
FIGURE 1. Gene expression, protein level, and stability of Tgl3p in the absence of LD. A, relative gene expression of TGL3 in wild type (WT) (black bar) and QM (gray bar) measured by RT-PCR. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation. B, protein analysis of Tgl3-Myc of total cell extracts from wild type and QM grown to the stationary phase. C, relative protein level of Tgl3-Myc of total cell extracts from wild type (black bar) and QM (gray bar) obtained by three Western blots was calculated using ImageJ program. D, Western blot analysis of Tgl3-Myc was performed with total cell extracts from wild type and QM grown for time periods as indicated after addition of 100 μg/ml cycloheximide to cells grown to mid-logarithmic phase. GAPDH was used as loading control. E, relative protein stability in wild type and QM obtained by three Western blots was calculated using the ImageJ program. Protein half-life is shown. Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

FIGURE 2. Localization and lipase activity of Tgl3p in the absence of LD. A, Western blot analysis of Tgl3-Myc in homogenate (Hom), 30,000 × g microsomes (M30), 40,000 × g microsomes (M40), cytosol (Cyt), and LD fraction (LD) from wild type (WT) and QM grown to the stationary phase. Primary antibodies were directed against the Myc tag, Wbp1p (ER marker), and GAPDH (cytosolic marker). Western blot analyses are representative of at least two independent experiments. B, fluorescence microscopy of PGal1-GFP-Tgl3 in wild type and QM grown to late logarithmic phase after induction with galactose for 4 h. Two different sections from the QM strain are shown. Size bar, 5 μm. C, analysis of TG lipase activity of LD and 30,000 × g ER fractions from wild type and QM overexpressing TGL3. Experiments were performed in triplicates and are representative of at least two independent experiments. Data are mean values with the respective deviation. DIC, differential interference contrast.
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FIGURE 3. Localization of Tgl3p in yeast strains lacking nonpolar lipid-synthesizing enzymes. A, Western blot analysis of Tgl3-Myc in homogenate and LD fractions from wild type (WT), lro1Δare1Δare2Δ, and dga1Δlro1Δ grown to the stationary phase. B, relative protein levels of Tgl3-Myc from total cell extracts of wild type (black bar), lro1Δare1Δare2Δ (gray bar), and dga1Δlro1Δ (light gray bar) obtained by three Western blots were calculated using ImageJ program. C, Western blot analysis of Tgl3-Myc with total cell extracts from lro1Δare1Δare2Δ and dga1Δlro1Δ grown for time periods as indicated after addition of 100 μg/ml cycloheximide to cells grown to the mid-logarithmic phase. D, relative protein stability in lro1Δare1Δare2Δ and dga1Δlro1Δ obtained by three Western blots was calculated using ImageJ program. Protein half-life is shown. E, Western blot analysis of Tgl3-Myc in homogenate (Hom), 30,000 × g microsomes (M30), 40,000 × g microsomes (M40), and cytosol (Cyt). Primary antibodies were directed against the Myc tag, Wbp1p (ER marker), GAPDH (cytosolic marker), Ayr1p (LD marker), and Erg1p (LD marker). Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

ity, and localization of Tgl3p in mutants lacking either TG or SE. Czabany et al. (36) had shown that the four nonpolar lipid-synthesizing enzymes Dga1p, Lro1p, Are1p, and Are2p contribute differently to LD formation. The important finding of these authors was that S. cerevisiae mutant strains lacking either TG or SE, respectively, were still able to form LD, which differed, however, in number, structure, lipid, and protein composition. In an lro1Δare1Δare2Δ strain where Dga1p was the only active acyltransferase, LD consisted entirely of TG, whereas in a dga1Δlro1Δ strain both SE synthases are actively producing SE as the only storage lipid of LD. In these strains, gene expression of TGL3 is only slightly altered compared with wild type (data not shown). However, Western blot analysis of Tgl3p revealed that the amount of the enzyme was reduced in dga1Δlro1Δ but not in lro1Δare1Δare2Δ (Fig. 3A). Quantification of Western blots revealed a reduction of total Tgl3p in dga1Δlro1Δ to 60% of wild type and lro1Δare1Δare2Δ (Fig. 3B). Interestingly, the relative amount of Tgl3p on LD was not changed in all mutants (Fig. 3A). The reduced protein level in the dga1Δlro1Δ strain suggested a reduced stability of Tgl3p in cells lacking TG. Indeed, Fig. 3, C and D, shows that degradation of Tgl3p in the dga1Δlro1Δ strain is similar to the QM. In contrast, Tgl3p is rather stable in an lro1Δare1Δare2Δ strain. The half-life of Tgl3p was 6 h in dga1Δlro1Δ and more than 9 h in lro1Δare1Δare2Δ (Fig. 3D).

Because experiments described above demonstrated a correlation of protein stability and localization of Tgl3p to the ER, we assumed that the subcellular distribution of Tgl3p was also affected in a yeast strain lacking TG. Indeed, a large portion of the lipase was found in the ER of a dga1Δlro1Δ strain that contains SE as the only nonpolar lipid in LD (Fig. 3E; M30 and M40 fractions), although Tgl3p was still present in LD. In the dga1Δlro1Δ strain, the enrichment of Tgl3p in microsomal fractions over the homogenate was similar to the ER marker protein Wbp1p. However, it has to be noted that the amount of LD in dga1Δlro1Δ is markedly lower than in wild type yeast cells (36). Thus, the low number of LD in this mutant and its altered nonpolar lipid composition may cause the altered subcellular localization of Tgl3p in dga1Δlro1Δ. In sharp contrast, Tgl3p was not enriched in microsomes from the lro1Δare1Δare2Δ strain that contains LD with TG as the only nonpolar lipid. In this mutant, the
subcellular distribution of Tgl3p was the same as in the wild type.

Gene Expression, Stability, and Localization of Tgl3p Variants Bearing Mutations in the Two Active Centers of the Enzyme—As described above, considerable amounts of Tgl3p are present in yeast cells lacking TG and even the storage compartment for nonpolar lipids, the LD. This finding raised the question as to the function of Tgl3p in the absence of its substrate. Rajakumari and Daum (25) demonstrated that Tgl3p not only acts as a lipase but also as an acyltransferase mediating acyl-CoA-dependent acylation of lysophospholipids. These two enzymatic activities are catalyzed by two independent active centers. Therefore, we speculated that the lack of TG and shift of Tgl3p from LD to the ER might favor the second function of the protein, the acyltransferase activity. To address this question, we tested Tgl3p variants bearing mutations in one or both of the active centers for gene expression, protein level, and localization. A lipase-defective mutant Tgl3S237A, an acyltransferase-defective Tgl3H298A, and a Tgl3S237A/H298A mutant bearing mutations in both active centers were analyzed. Quantitative RT-PCR analysis revealed no significant changes in mRNA levels of yeast strains expressing the different variants of TGL3 compared with wild type (Fig. 4A). Furthermore, protein levels of mutated Tgl3p proteins were not changed (Fig. 4B). Finally, none of the tested mutations at the active centers of Tgl3p affected localization of the enzyme to LD, because signals of HA-Tgl3p variants were still highly enriched in LD fractions (Fig. 4C). Concomitantly, the occurrence of Tgl3p variants in microsomal fractions was not affected (Fig. 4D). Thus, we conclude that changes in the enzymatic activities of Tgl3p introduced by the above-mentioned mutations did not influence formation and localization of the enzyme.

TG Substrate Availability Affects Contribution of Tgl3p to Phospholipid Synthesis—Because Tgl3p appears to also play a role in phospholipid metabolism of S. cerevisiae (25), we tested its possible contribution to phospholipid synthesis in the absence of nonpolar lipids. To address this question, we performed phospholipid analysis of different mutants. Fig. 5A shows the amount of total phospholipids/mg of protein of total cell extracts from wild type (WT) and mutants with additional deletion of TGL3 grown to the stationary phase. The wild type (45 mg of phospholipids/mg of protein) was set at 100%. B, analysis of lysophosphatidylethanolamine acyltransferase activity in 30,000 g ER fractions from QM and QM tgl3 mutant. Assays were performed in triplicate from at least two independent biological samples. Data are mean values with the respective deviation. C, relative amount of DG from wild type and QM with additional deletion of TGL3 in cells grown to the stationary phase. Data are mean values of three independent experiments with respective mean deviations. Wild type was set at 100%.

FIGURE 4. Gene expression, protein level, and localization of Tgl3p in different mutants. A, relative gene expression of TGL3WT, tgl3S237A, tgl3H298A, and tgl3S237A/H298A was measured by RT-PCR. TGL3WT was set at 1. Data are mean values from three independent experiments with the respective deviations. B, protein analysis of Tgl3WT, Tgl3S237A, Tgl3H298A, and Tgl3S237A/H298A from total cell extracts grown to stationary phase. GAPDH was used as loading control. C, Western blot analysis of Tgl3WT, Tgl3S237A, Tgl3H298A, and Tgl3S237A/H298A in homogenate (Hom) and LD fractions from cells grown to the stationary phase. D, Western blot analysis of Tgl3p and variants in homogenate (Hom), 30,000 × g microsomes (M30), and 40,000 × g microsomes (M40). Primary antibodies were directed against the HA tag, Wbp1p (ER marker), GAPDH (cytosolic marker), Ayr1p (LD marker), and Erg6p (LD marker). Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

FIGURE 5. Lipid analysis of yeast strains lacking major nonpolar lipid-synthesizing enzymes. A, relative amounts of total phospholipids/mg of protein of total cell extracts from wild type (WT) and mutants with additional deletion of TGL3 grown to the stationary phase. Data are mean values of three independent experiments with respective deviations. The wild type (45 mg of phospholipids/mg of protein) was set at 100%. B, analysis of lysophosphatidylethanolamine acyltransferase activity in 30,000 × g ER fractions from QM and QM tgl3 mutant. Assays were performed in triplicate from at least two independent biological samples. Data are mean values with the respective deviation. C, relative amount of DG from wild type and QM with additional deletion of TGL3 in cells grown to the stationary phase. Data are mean values of three independent experiments with respective mean deviations. Wild type was set at 100%.
because the amount of phospholipids was reduced to 80% of wild type in a \( tgl3\Delta \) mutant. Interestingly, however, deletion of \( TGL3 \) in the QM background did not change the amount of total phospholipids. We also tested \textit{in vitro} acyltransferase activity of Tgl3p in a 30,000 \( \times \) g ER fraction from QM and QM\( tgl3\Delta \). Fig. 5B shows that deletion of \( TGL3 \) in the QM background did not change lysophosphatidylethanolamine acyltransferase activity. Similar measurements with wild type and \( tgl3\Delta \) did not show any differences either (data not shown). These results indicate that Tgl3p does not play a significant role in phospholipid synthesis when located to the ER. Apparently, Tgl3p requires the presence of TG to contribute efficiently to phospholipid synthesis. To address this question in more detail, we determined the amount of total phospholipids in \( lro1\Delta are1\Delta are2\Delta \) and \( dga1\Delta lro1\Delta \) strains with and without additional deletion of \( TGL3 \). Deletion of \( TGL3 \) resulted in a slightly reduced phospholipid level in the \( lro1\Delta are1\Delta are2\Delta \) background when LD contained TG but not in a \( dga1\Delta lro1\Delta \) strain where SE are the only nonpolar lipid components of LD. To further investigate a possible role of Tgl3p in phospholipid synthesis, we analyzed the phospholipid composition of wild type, QM, \( lro1\Delta are1\Delta are2\Delta \), and \( dga1\Delta lro1\Delta \) strains with an additional deletion of \( TGL3 \), respectively (Table 4). These analyses revealed that the phospholipid composition of wild type and \( lro1\Delta are1\Delta are2\Delta \) were clearly different from \( dga1\Delta lro1\Delta \) and QM strain. In \( dga1\Delta lro1\Delta \) and the QM, the amount of phosphatidylinositol and phosphatidylserine is markedly reduced compared with wild type and \( lro1\Delta are1\Delta are2\Delta \). In contrast, phosphatidylethanolamine and phosphatidylcholine were increased in \( dga1\Delta lro1\Delta \) and QM strain, respectively. A slight increase of lysophosphatidyls were measured when \( TGL3 \) was deleted in wild type and \( lro1\Delta are1\Delta are2\Delta \) background. In contrast, no enrichment of lysophosphatidyls was observed in \( dga1\Delta lro1\Delta \) and the QM. These data indicate that Tgl3p requires LD with both TG and SE or at least the presence of TG to serve as a lysophospholipid acyltransferase.

Kurat et al. (22) demonstrated that Tgl3p preferentially hydrolyzes TG but also exhibits minor DG lipolytic activity. Therefore, we speculated that Tgl3p may act as a DG lipase in the absence of TG. Fig. 5C shows the amounts of DG in strains lacking \( TGL3 \) in wild type and QM background. Compared with wild type and \( tgl3\Delta \), QM and QM\( tgl3\Delta \) strains show higher levels of DG. The increased amount of DG in these mutants can be explained by the lack of TG synthesis, rendering strains unable to convert DG to TG. Additionally, the fatty acid composition of DG is slightly altered in the QM compared with wild type (data not shown). However, deletion of \( TGL3 \) in the QM background did not further affect the DG level (Fig. 5C) and the fatty acid composition of DG (data not shown). Thus, Tgl3p does not gain relevant DG hydrolytic activity \textit{in vivo} when TG is missing. Altogether, Tgl3p appears to be rather inactive in the absence of LD and after a shift to the ER. Thus, the ER may be regarded as a parking lot for the yeast TG lipase Tgl3p.

**DISCUSSION**

The major TG lipase Tgl3p from \textit{S. cerevisiae} LD plays a critical role in TG mobilization but also contributes to phospholipid metabolism (20, 23). Although the biochemistry of this enzyme has been studied in some detail (25), little is known about the regulation of Tgl3p activity. Here, we provide some insight into the regulatory aspects of the TG metabolic network in \textit{S. cerevisiae} with emphasis on the role of Tgl3p. As possible mechanisms regulating the activity of Tgl3p, transcriptional and translational control, protein stability, and subcellular localization of the enzyme were anticipated. As another very important regulatory aspect, the substrate availability was considered as well as a possible feedback control by the products formed. Finally, post-translational modifications or direct inhibitory or stimulating effects on the enzyme level may play a role for the activity of Tgl3p.

In this study, we show that regulation of Tgl3p activity on the gene expression level is of minor importance. Our data demonstrated only minor changes of \( TGL3 \) expression in wild type and mutants lacking nonpolar lipids and consequently LD (see Fig. 1A) or bearing mutations in the active centers of the enzyme (see Fig. 4A).

A more important regulatory mechanism is the protein stability of the TG lipase Tgl3p especially in the absence of LD (see Fig. 1). Sorger et al. (19) had demonstrated that squalene epoxidase Erg1p, another protein that is dually located to LD and the ER, is stable only when located to LD. In the absence of LD, \textit{i.e.} in a \( dga1\Delta lro1\Delta are1\Delta are2\Delta \) mutant, the stability of Erg1p was strongly compromised. A stabilizing effect of LD on the polypeptide was suggested. Thus, stabilization of proteins by association with the LD surface membrane may not only be an exclusive effect for one or two proteins such as Tgl3p and Erg1p, but probably is a more general phenomenon. With this regard, the topology of LD proteins and their assembly into the
surface monolayer of LD may play an important role. Additionally, the absence or presence of TG, the major substrate of Tgl3p, seems to cause marked changes in protein stability (see Fig. 3). Previous studies had already shown that some typical LD proteins are located to the ER in the absence of LD (10, 19). These observations supported the view that LD and ER are closely related subcellular fractions. Thus, it was not surprising that also Tgl3p was localized to the ER in a QM. The dual localization of Tgl3p to LD and the ER in wild type (see Fig. 2A) has not been explicitly shown before but is in line with other reports about LD proteins (37). More surprising was the finding that the absence or presence of the major lipase substrate TG affected the distribution of Tgl3p between LD and ER (see Fig. 3E). LD from yeast strains lacking one or more nonpolar lipid-synthesizing enzymes vary in lipid composition, structure, size, and number (36). Moreover, it was reported that the lipid composition of LD affects the protein equipment of the organelle. Thus, the absence of TG appears to have such an effect on Tgl3p. It has to be noted, however, that TG are not essential for Tgl3p localization to LD, but in the absence of TG, the number and size of LD are significantly reduced, which causes a sort of overflow of the enzyme to the ER.

As described above, lack of LD or depletion of TG causes complete or partial re-localization of Tgl3p to the ER. This re-localization may be explained by the functional and biosynthetic link of LD and the ER. It appears that Tgl3p is retained to the ER when LD are missing or insufficiently equipped with TG. Entire or partial re-localization of Tgl3p to the ER always results in a loss of protein stability. This effect may be due to the inappropriate embedding and altered topology of Tgl3p in the ER bilayer membrane. Our preliminary results indicate that Tgl3p located to the ER becomes more accessible to proteolytic digestion than in the monolayer membrane of LD.

Tgl3p was shown to act not only as TG lipase but also as a lysophospholipid acyltransferase (25). The two activities of the enzyme are independent of each other and catalyzed by two distinct active centers. This finding led us to speculate that these two enzymatic activities might also be regulated independently. Most surprisingly, our results demonstrated that Tgl3p needs to be located to LD with TG present to contribute to phospholipid synthesis in vivo (see Fig. 5A and Table 4). As the presence of the lipase substrate TG seems to be important also for the acyltransferase activity, we speculate that TG degradation and lysophospholipid acylation catalyzed by Tgl3p are linked processes. In vitro Tgl3p utilizes acyl-CoAs for efficient acylation of lysophosphatidylethanolamine, indicating that fatty acyl activation is required for this phospholipid biosynthetic route. Indeed, such fatty acid-activating enzymes are present on the surface of LD, which might contribute to this second activity of Tgl3p (5, 6). Additionally, the two other TG lipases of the yeast, Tgl4p and Tgl5p, might also play a role in providing fatty acids for phospholipid biosynthesis on LD. Similar to Tgl3p, these two lipases are lysophospholipid acyltransferases (24, 25). Furthermore, interaction between LD and the ER may be required for a concerted action in phospholipid biosynthesis (10).

In summary, our results demonstrate that TG substrate limitation in the yeast causes changes in Tgl3p stability, gene expression, and localization. Moreover, a link between TG lipolysis and the capacity of Tgl3p to perform acyltransferase reactions was shown. These findings are novel facets in the regulatory network of nonpolar lipid metabolism.

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