The TGL2 Gene of Saccharomyces cerevisiae Encodes an Active Acylglycerol Lipase Located in the Mitochondria*

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Hye Jin Ham, Hyun Joo Rho, Seung Koo Shin1, and Hye-Joo Yoon2

From the Bio-Nanotechnology Center, Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea

The Saccharomyces cerevisiae Tgl2 protein shows sequence homology to Pseudomonas triacylglycerol (TAG) lipases, but its role in the yeast lipid metabolism is not known. Using hemagglutinin-tagged Tgl2p purified from yeast, we report that this protein carries a significant lipolytic activity toward long-chain TAG. Importantly, mutant hemagglutinin-Tgl2pS144A, which contains alanine 144 in place of serine 144 in the lipase consensus sequence (G/A)XXG exhibits no such activity. Although cellular TAG hydrolysis is reduced in the tgl2 deletion mutant, overproduction of Tgl2p in this mutant leads to an increase in TAG degradation in the presence of fatty acid synthesis inhibitor cerulenin, but that of Tgl2pS144A does not. This result demonstrates the lipolytic function of Tgl2p in yeast. Although other yeast TAG lipases are localized to lipid particles, Tgl2p is enriched in the mitochondria. The mitochondrial fraction purified from the TGL2-overexpressing yeast shows a strong lipolytic activity, which was absent in the tgl2 deletion mutant. Therefore, we conclude that Tgl2p is a functional lipase of the yeast mitochondria. By analyzing phenotypic effects of TGL2-deficient yeast, we also find that lipolysis-competent Tgl2p is required for the viability of cells treated with antimitotic drug. The addition of oleic acid, the product of Tgl2p-catalyzed lipolysis, fully complements the antimitotic drug sensitivity of the tgl2 null mutation. Thus, we propose that the mitochondrial Tgl2p-dependent lipolysis is crucial for the survival of cells under antimitotic drug treatment.

Lipases or triacylglycerol (TAG)3 hydrolases (EC 3.1.1.3) are ubiquitous enzymes found from bacteria to humans and catalyze the hydrolysis (and synthesis) of ester bonds in relatively long-chain acylglycerols, comprising a subclass of the esterases. Lipase-catalyzed hydrolysis of TAG primarily produces fatty acid and diacylglycerol (DAG). DAG can be further degraded to fatty acid, monoacylglycerol (MAG), and/or glycerol. Both TAG and its degradation products perform important functions in an organism, and the regulation of lipid metabolism is critical for growth and proliferation of all types of cells. Perturbed lipid homeostasis has been linked to lipotoxic cell death pathways and various metabolic disorders (1, 2).

The catalytic center of lipases consists of an S...D/E...H triad which is found in the active site of α/β hydrolyase fold enzymes and serine proteases, although the order of the three residues is different (3–5). In particular, serine is essential for catalysis and participates, with aspartic acid/glutamic acid and histidine, in the charge relay system. Serine invariably occurs in a highly conserved sequence, GXXG, in which the first glycine residue is replaced by alanine in some lipases, commonly in Bacillus lipases (3–7).

In the yeast Saccharomyces cerevisiae, TAG is mobilized by three lipases, Tgl3p, Tgl4p, and Tgl5p (8–10). Interestingly, all three proteins are embedded in lipid particles, which store energy in the form of neutral lipids, predominantly TAGs and sterol esters. In addition to being simple storage sites for energy, lipid particles play diverse physiological roles, including communication and transfer of molecules through organelar associations (11). Because the S. cerevisiae lipid particles contain many enzymes involved in the metabolism of TAGs and sterol esters, it has now become clear that these particles actively participate in lipid metabolism (12).

Tgl3p, Tgl4p, and Tgl5p do not show homology to other lipases identified so far, except that all three carry the lipase consensus sequence (G/A)XXG. Serine 315 in this sequence is essential for the catalytic activity of Tgl4p, the functional ortholog of mammalian adipose TAG lipase (10). Most recently, it has been reported that Tgl4p is activated by the cyclin-dependent kinase Cdk1/Cdc28 via phosphorylation at threonine 675 and serine 890 and that lipolysis catalyzed by the phosphorylated Tgl4p contributes to the early bud formation in late G1 phase of the cell cycle (13). Tgl5p may act synergistically with Tgl4p and play a minor or regulatory role in TAG hydrolysis, because single deletion of TGL5 results in no reduction in TAG degradation in vivo in the presence of the fatty acid synthesis inhibitor cerulenin, whereas the tgl4Δtgs5Δ double mutant exhibits significant reduction in this activity compared with the tgl4Δ single mutant (9). Tgl3p carries relatively high lipolytic activity among the three lipases, and together with Tgl4p, it constitutes the majority of lipolytic activity in lipid particles and whole cells (8–10).

Meanwhile, a TAG lipase activity has been detected from the mitochondrial fraction of S. cerevisiae (14, 15). None of the known yeast lipases can account for such mitochondrial activity, as evidenced by their subcellular localization to lipid particles. A mitochondrial protein carrying the lipolytic activity still needs to be identified.

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1 To whom correspondence may be addressed. Fax: 82-054-279-3399; E-mail: skshin@postech.ac.kr.
2 To whom correspondence may be addressed. Fax: 82-054-279-3399; E-mail: hjyoon@postech.ac.kr.
3 The abbreviations used are: TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; HA, hemagglutinin.
Mitochondrial Tgl2 Lipase

The *S. cerevisiae* Tgl2p is considered to be a potential TAG lipase based on the sequence homology to *Pseudomonas* lipases (16). Initially, TGL2 has been isolated as a yeast gene capable of complementing the *Escherichia coli* DAG kinase mutation, which results in lethal accumulation of DAG in the absence of hydroquinone β-d-glucopyranoside arbutin (16). Because the expression of TGL2 in this mutant produces *E. coli* lysates harboring no DAG kinase activity but lipolytic activity toward relatively short-chain TAGs and DAGs, it is plausible that TGL2 suppresses the toxicity via the DAG hydrolytic activity of its gene product (16). However, no lipolytic activity of Tgl2p has been demonstrated in *S. cerevisiae*. The *S. cerevisiae* DAG kinase that catalyzes the formation of phosphatidate from DAG has recently been identified (17, 18).

To unravel the biological function of Tgl2p, we conducted enzymatic analysis of the protein and phenotypic characterization of its gene deletion and overexpression strains. Here, we report the identification of Tgl2p as an active lipase of the yeast mitochondria based on the data obtained from subcellular fractionation and *in vivo* and *in vitro* experiments on lipid hydrolysis. By phenotypic analysis of TGL2-deficient yeast, we also demonstrate that Tgl2p-catalyzed lipolysis or its product oleic acid is required for the viability of cells treated with tubulin-targeting agents.

**EXPERIMENTAL PROCEDURES**

**Construction of tgl2Δ Null Strain**—The haploid tgl2Δ null mutant (YHY058d2 [MATa ade2–101 his3–Δ200 leu2–Δ1 lys2–801 trpl–Δ63 ura3–52 tgl2Δ::URA3 CFIII (CEN3.LYPH983 HIS3 Slip11)]) was constructed as follows. A PCR product spanning TGL2, including 509-bp upstream and 270-bp downstream sequences, was cloned into the BamHI site of pRS314 (19). The 458-bp Ncol-AatII fragment of TGL2 was replaced with a 1.1-kb HindIII fragment containing the yeast *URA3* gene, and the resulting plasmid pRS314-ΔTGL2 was digested with BamHI and transformed into diploid strain YHY399 (20). The resulting diploid strain bearing one disrupted copy of TGL2 was sporulated, and the spores were analyzed. The tgl2Δ deletion was verified by PCR with genomic DNA of the haploid derivatives.

**Plasmids and Cloning**—To generate plasmid pYNO4-HA-TGL2 expressing the hemagglutinin (HA)-tagged TGL2 under the control of the *GAL1* promoter, a PCR product containing the TGL2 open reading frame with an EcoRI site before the start codon and a ClaI site after the stop codon was cloned into the EcoRI and ClaI sites of pYNO4. Plasmid pYNO4 was derived from pRS314 (CEN6, TRP1) by inserting into its KpnI and SacI sites a 1-kb cassette carrying the *GAL1/10* promoter, triple HA repeats, and multiple cloning sites. The amino acid sequence of a peptide linked to the N terminus of Tgl2p is MVGYPYDVP-repeats, and multiple cloning sites. The amino acid sequence of the pRS314 (19). The 458-bp Ncol-AatII fragment of TGL2 was replaced with a 1.1-kb HindIII fragment containing the yeast *URA3* gene, and the resulting plasmid pRS314-ΔTGL2 was digested with BamHI and transformed into diploid strain YHY399 (20). The resulting diploid strain bearing one disrupted copy of TGL2 was sporulated, and the spores were analyzed. The tgl2Δ deletion was verified by PCR with genomic DNA of the haploid derivatives.

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**Lipase Assay in Vitro**—Lipase assay buffer was prepared by mixing 150 µl of 50 mM Tris-HCl (pH 8.0) and 20 µl of a bovine serum albumin solution (20 mg/ml). Lipids (TAG, DAG, MAG, cardiolipin, or phosphatidylcholine) were added to a final concentration of 2–4 mM. The mixture was sonicated at 37 °C until the component concentration of 2–4 mM. The mixture was sonicated at 37 °C until the solution became cloudy (≥4 min). After the addition of 50 µl of 200 mM MgCl₂, the mixture was pretreated at 37 °C (total volume, 220 µl). Lipase reaction was initiated by mixing 220 µl of pretreated solution and 200 µl of protein preparation (mitochondrial fractions or anti-HA affinity-purified proteins). An aliquot was taken out at the start (time 0) to determine the initial lipid concentration in the reaction mixture. After incubation for 1 h at 37 °C, lipids were immediately separated by TLC. If necessary, lipids were extracted by the addition of 1 ml of chloroform before TLC separation.
Lipid Analysis—Routine analysis of lipid samples (TAG, DAG, or MAG) was done as described previously (22) with modifications. Lipids were applied as 2-μl spots on Silica Gel 60 G plates (catalog number 5721, Merck) and separated by TLC in the solvent mixture of hexane/diethyl ether/acetic acid (50: 50:1, v/v/v). Lipid spots were identified under a UV lamp after spraying a primuline solution (5 mg in 100 ml of acetone/water (80:20, v/v)) or 50 mM CuCl₂ solution. Analysis of phospholipids (cardiolipin or phosphatidylcholine) was done by following a published procedure (23). Lipase activity was measured by comparing the amount of lipid degraded after 1 h of incubation relative to the amount at time 0. Known amounts of lipid were run on the TLC plates to obtain the linear standard curve, and the amount of each lipid spot after 1 h of incubation was determined from the curve. The fluorescence imaging system was employed to integrate lipid spot intensities. TAGs (triolein, tripalmitin, tricaprylin, and tributyrin), DAGs (diolein, dipalmitin, and dicaprylin), MAGs (monoleoin, monopalmitin, and monocabrylin), and phospholipids (cardiolipin and phosphatidylcholine) were purchased from Sigma.

Analysis of TAG Degradation in Vivo—To obtain samples grown in the presence or absence of cerulenin, tgl2Δ cells (strain YHY058d2) and isogenic wild-type cells were grown in synthetic glucose medium lacking 10 μg/ml cerulenin (dissolved in ethanol) or ethanol alone. Meanwhile, tgl2Δ cells harboring pYN04, pYN04-4HA-TGL2, or pYN04-HA-TGL2S144A were grown in synthetic glucose medium lacking tryptoophan at 30 °C overnight and inoculated to A₆₀₀nm = 2 in the fresh medium supplemented with 10 μg/ml cerulenin (dissolved in ethanol) or ethanol alone. Meanwhile, tgl2Δ cells harboring pYN04, pYN04-4HA-TGL2, or pYN04-HA-TGL2S144A were grown in synthetic glucose medium lacking tryptoophan at 30 °C overnight and inoculated to A₆₀₀nm = 3 in tryptophan-free, synthetic galactose medium supplemented with 10 μg/ml cerulenin or ethanol. Yeast growth was monitored by measuring the absorbance at 600 nm. At the times indicated, 10-ml aliquots were pelleted, washed with distilled H₂O, instantly frozen in liquid nitrogen, and stored at −70 °C. Cells were resuspended in 0.5 ml of sorbitol buffer (40 mM potassium phosphate, pH 6.5, 0.5 mM MgCl₂, 1.2 mM sorbitol) and incubated with 1 μl of β-mercaptoethanol and 30 μl of 5 mg/ml zymolyase 100T (catalog number 120493, Seikagaku Corp.) for 15 min at 30 °C. After spheroplast disruption, lipids were harvested on the TLC plates to obtain the linear standard curve, and relative to the amount at time 0. Known amounts of lipid were loaded onto a sucrose gradient to remove the contaminated lipids (cardiolipin or phosphatidylcholine) was done by following a published procedure (23). Lipase activity was measured by adding mounting solution (1 mg/ml p-phenylene-diamine, 90% glycerol) containing 50 ng/ml 4′,6-diamidino-2′-phenylindole, slides were examined with a Nikon Eclipse 80i microscope using a ×100 oil immersion objective. Digital images were captured with a Nikon DMX 1200F camera.

Benomyl Sensitivity Test—Yeast cells, tgl1Δ, tgl2Δ, and isogenic wild-type cells were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) medium to A₆₀₀nm = 0.8. Aliquots of each culture were serially diluted in 10-fold steps up to a 10⁴ dilution. Each dilution (3 μl) was spotted on YPD agar plates containing DMSO (control), benomyl (85 μM), fatty acid (2 mM), or benomyl (85 μM) plus fatty acid (2 mM). Fatty acid (butyric, palmitic, palmitoleic, stearic, or oleic acid) was added to the other components as a 20:1 (v/v) mixture of fatty acid/ Tween 20TM (Sigma) after vortexing. The plates were photographed after a 3-day incubation at 30 °C. Effects of benomyl on the growth of tgl2Δ cells harboring pRS314, pRS314-796-HA-TGL2, or pRS314-796-HA-TGL2S144A were tested by growing cells in synthetic glucose medium lacking tryptophan and spotting each dilution on agar plates of the same medium containing DMSO, benomyl (100 μM), or benomyl (100 μM) plus oleic acid (1 or 2 mM). The plates were incubated for 4 days at 30 °C.

RESULTS

TAG Lipase Activity of Tgl2p—To determine the lipolytic activity of Tgl2p, HA-tagged Tgl2p was purified close to homogeneity from a tgl2Δ strain overexpressing this fusion protein. The anti-HA affinity-purified fraction exclusively contained Tgl2p, not any other protein tightly associated with it, and serine 144 of the Tgl2p is conserved from a published procedure (23). Lipase activity was measured by comparing the amount of lipid degraded after 1 h of incubation relative to the amount at time 0. Known amounts of lipid were run on the TLC plates to obtain the linear standard curve, and the amount of each lipid spot after 1 h of incubation was determined from the curve. The fluorescence imaging system was employed to integrate lipid spot intensities. TAGs (triolein, tripalmitin, tricaprylin, and tributyrin), DAGs (diolein, dipalmitin, and dicaprylin), MAGs (monoleoin, monopalmitin, and monocabrylin), and phospholipids (cardiolipin and phosphatidylcholine) were purchased from Sigma.

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Purification of Yeast Mitochondria—Crude mitochondrial fractions were prepared by the published method (25) and loaded onto a sucrose gradient to remove the contaminated endoplasmic reticulum and vacuole (26). After centrifugation at 134,000 × g in a Beckman SW41 Ti swinging bucket rotor for 1 h at 2 °C, pure mitochondria were recovered from the 32%/60% interface of the gradient. The protein concentration was adjusted to 10 mg/ml after pelleting pure mitochondrial fraction at 10,000 × g.

Immunofluorescence Microscopy—Yeast cells were formaldehyde-fixed and prepared for indirect immunofluorescence (27). After adding mounting solution (1 mg/ml p-phenylenediamine, 90% glycerol) containing 50 ng/ml 4′,6-diamidino-2′-phenylindole, slides were examined with a Nikon Eclipse 80i microscope using a ×100 oil immersion objective. Digital images were captured with a Nikon DMX 1200F camera.
immunoblot analysis confirmed that the relative amounts of HA-Tgl2p and HA-Tgl2pS144A were almost identical in our protein extracts with or without cerulenin treatment (data not shown). Significant change in the growth rate was not observed in the presence of cerulenin, although significant change in the growth rate was not observed from whole cell lysates (data not shown). HA-Tgl2p and HA-dTgl2p (proteolytic fragment of HA-Tgl2p) are indicated by arrows. The molecular sizes are shown on the left. HA-Tgl2p-specific TAG lipase activity. Anti-HA affinity-purified proteins as shown in A were assayed for TAG lipase activity using 3.6 μM triolein as substrate. Results represent data (mean ± S.D.) obtained from three separate measurements. ND, not detected.

**FIGURE 1.** **TAG lipase activity of purified HA-Tgl2p.** A, affinity isolation of HA-Tgl2 fusion protein. Tgl2p epitope-tagged, overexpressed, and purified as described under "Experimental Procedures" was subjected to SDS-PAGE and visualized by Sypro Ruby staining. The haploid tgl2Δ strain (YHY058d2) harboring the control plasmid pYN04 (lane 1), pYN04-HA-TGL2 (lane 2), or pYN04-HA-TGL2S144A (lane 3) was used to prepare anti-HA affinity fraction from whole cell lysates (~ 3 mg of protein extracts). HA-Tgl2p and HA-dTgl2p (proteolytic fragment of HA-Tgl2p) are indicated by arrows. The molecular sizes are shown on the left. B, HA-Tgl2p-specific TAG lipase activity. Anti-HA affinity-purified proteins as shown in A were assayed for TAG lipase activity using 3.6 μM triolein as substrate. Results represent data (mean ± S.D.) obtained from three separate measurements. ND, not detected.

**FIGURE 2.** **Tgl2p-specific degradation of TAG in vivo.** Growth curves (A and C) and cellular TAG levels (B and D) of tgl2Δ (A and B; open and solid squares, dashed and dotted line), isogenic wild-type (A and B; open and solid circles, solid line), and tgl2Δ strain transformed with pYN04 (C and D; open and solid squares, dashed and dotted line), pYN04-HA-TGL2 (C and D; open and solid triangles, solid line), or pYN04-HA-TGL2S144A (C and D; open and solid triangles, dotted line) are shown. Precultured cells were inoculated into fresh medium at time 0 and grown in the presence of ethanol (open symbols) or 10 μg/ml cerulenin dissolved in ethanol (solid symbols). Lipids were extracted and analyzed at the indicated time points as described under Experimental Procedures. Cellular TAG levels are presented as relative abundance of TAG per cell density (A000 nm; OD600 nm). TAG/A000 nm at time 0 is set at 100%. Data represent means and S.D. (error bars) of three independent experiments.

**FIGURE 3.** **Enzymatic properties of the Tgl2 lipase.** Dependence on the acyl chain length of substrate TAG. A, temperature (B), pH (C), and divalent metal ion or EDTA (D) was determined using affinity-purified HA-Tgl2p. In D, the lipase assay buffer contained 25 mM MgCl2 (Mg2+2), 25 mM CaCl2 (Ca2+), water (none), or 50 mM EDTA (EDTA). The lipolytic activity toward tributyrin (C4:0) at pH 8 and 37 °C in the presence of 25 mM Mg2+ is set at 100%. This corresponds to the specific activity of 3.2 ± 0.2 μmol mg−1 min−1. Data represent means and S.D. (error bars) of three independent experiments.
Tgl2p showed optimal activity toward tributyrin, further enzymatic analyses were performed using tributyrin as substrate.

To determine the temperature optimum, tributyrin hydrolysis was measured at 25, 37, 55, and 70 °C (Fig. 3B). Although the activity reached a maximum at 37 °C, which was set at 100%, Tgl2p was still active at high temperatures, retaining 40 and 39% activity at 55 and 70 °C, respectively. Lipases are known to be stable at high temperatures. At 25 °C, Tgl2p showed 50% activity. Regarding the pH optimum, Tgl2p was most active at pH 8, whereas it maintained more than 40% activity at pH 6 (53%) and pH 10 (42%) (Fig. 3C).

Next, the dependence on the divalent metal ion was monitored by replacing Mg²⁺ in the reaction mixture with Ca²⁺, Cu²⁺, and Zn²⁺. In comparison with the MgCl₂-containing sample, the tributyrin hydrolytic activity slightly increased by ~20% in the presence of CaCl₂ (Fig. 3D). In the cases of CuCl₂ and ZnCl₂, we were unable to measure lipolytic activity because precipitates were formed immediately upon the addition of these salts. Intriguingly, there was little need for divalent cations in the Tgl2 lipase activity. The addition of EDTA up to 50 mM did not abolish this activity (Fig. 3D).

Mitochondrial Localization and Substrate Specificity of Tgl2p—Subcellular location of Tgl2p is of interest because all three yeast lipases, Tgl3p, Tgl4p, and Tgl5p, are localized to the same organelle, the lipid particle. Using anti-HA immunofluorescence microscopy, we localized Tgl2p to the mitochondria after overexpressing the gene from the GAL1 promoter. It appeared that Tgl2p co-localized with the mitochondrial DNA as shown by the overlap of HA-Tgl2p staining with DNA staining excluding the nuclear DNA (Fig. 4A). The staining pattern of the mutant HA-Tgl2p (S144A) was identical to that of HA-Tgl2p (data not shown). We were unable to localize Tgl2p expressed from the endogenous TGL2 promoter, most likely due to its low abundance (data not shown). Recently, Tgl2p has been localized to the mitochondria using large scale cloning under the control of the moderately strong TEF1 promoter, green fluorescent protein tagging, and high resolution microscopy (31).

The mitochondrial localization of Tgl2p was confirmed by immunoblot analysis of subcellular fractions as shown in Fig. 4B. We isolated pure mitochondria with high yield from the crude mitochondrial fraction through a three-step sucrose gradient. In addition to the abundant Tgl2p obtained by galactose induction, we were able to detect the low level Tgl2p expressed from its native promoter in the pure mitochondria (Fig. 4B, lane 3). Small amounts of Tgl2p were also present in the cytosol and microsome when the protein was highly produced from the GAL1 promoter (Fig. 4B, lanes 4 and 5). This appears to be an artifact of overproduction because Tgl2p in its endogenous level was exclusively recovered with pure mitochondria just like the mitochondrial marker protein porin (Fig. 4B, lane 3). The S144A mutation did not cause mislocalization of Tgl2p (data not shown). In a separate experiment, we confirmed that Tgl2p was not present in the lipid particle or vacuole (data not shown).

As described above, the mitochondrial fraction of S. cerevisiae carries lipolytic activity (14, 15). The mitochondrial lipolytic activity was not detectable in tgl2Δ cells, but expression of HA-TGL2 from the native TGL2 promoter resulted in a specific activity of 0.4 ± 0.1 nmol mg⁻¹ min⁻¹ (Table 1). We were unable to recognize HA-Tgl2p produced from its endogenous promoter by visualizing mitochondrial proteins with Sypro Ruby staining even after anti-HA affinity isolation (Fig. 5, lanes A).
Mitochondrial Tgl2 Lipase

2 and 5). It should be noted that the endogenous level of HA-Tgl2p was seen only from immunoblots using highly sensitive enhanced chemiluminescence, which could detect femtomolar amounts of antigen (e.g. see Fig. 4B). Overexpression of HA-TGL2 led to ~50-fold amplification of the mitochondrial lipolytic activity, whereas that of HA-TGL2S144A showed no increase in the activity (Table 1). As shown in Fig. 5, HA-Tgl2p was significantly overproduced and accumulated in the mitochondria following galactose induction. To our surprise, the lipolytic activity (~13,000 nmol mg⁻¹ min⁻¹) of HA-Tgl2p purified from the mitochondrial fraction was enhanced by a factor of ~22 in comparison with that (~600 ± 60 nmol mg⁻¹ min⁻¹) purified from the whole cell lysate (Fig. 1B and Table 1). Mutant Tgl2pS144A isolated from the mitochondria was completely inactive (Table 1).

Although the mitochondrial Tgl2p exhibits a strong TAG hydrolytic activity toward long-chain TAG, this enzyme may have other substrates because TAGs are enriched in lipid particles and not in mitochondria. It is possible that mitochondrial phospholipids, such as cardiolipin, may be a physiological substrate for the Tgl2 lipase. Thus, we assayed various lipids for substrate specificity of the Tgl2 lipase purified from the mitochondrial fraction (Table 2). Notably, Tgl2p was incapable of hydrolyzing cardiolipin or phosphatidylcholine. Of the acyl-glycerols tested, Tgl2 lipase showed strong lipolytic activity toward DAGs and TAGs as well as MAGs, but was completely inactive toward MAGs (32, 33). None of these lipids were hydrolyzed by mutant Tgl2pS144A isolated from the mitochondria (data not shown). Taken together, our results imply that Tgl2p purified from the mitochondria possesses a strong lipase activity.

Phenotypic Effects of the tgl2Δ Mutation—The single null mutant tgl3Δ, tgl4Δ, or tgl5Δ, and even the triple mutant tgl3Δtgl4Δtgl5Δ, grow like wild type (9). Similarly, the TGL2 gene is neither essential nor important for cell growth (Fig. 2A). Deletion of TGL2 resulted in no growth defect in rich (YPD) or minimal medium at 16, 30, and 37 °C. No cold or temperature sensitivity was observed for tgl2Δ cells. Also, yeast growth on galactose or glycerol was not affected by the deletion (data not shown).

In a search for phenotypes associated with TGL2-deficient yeast, we found that the tgl2Δ mutant was more sensitive than the wild type to the anti-microtubule drug, benomyl (Fig. 6A). This phenotype was also confirmed by using another anti-microtubule drug, nocodazole (data not shown). As a control, tgl1Δ cells defective for sterol ester hydrolysis were also tested for the benomyl sensitivity. Tgl1p produces sterols and fatty acids by mobilizing sterol esters in the lipid particle (34). Similarly to the wild-type cells, tgl1Δ cells were not hypersensitive to benomyl (Fig. 6A). The benomyl-sensitive phenotype of the tgl2Δ mutation was rescued by expressing HA-TGL2 from the endogenous promoter. No suppression was observed by introducing the lipolysis-defective HA-TGL2S144A gene (Fig. 6B). Apparently, Tgl2 lipase is not required for the microtubule assembly or stability, because tgl2Δ cells exhibit little activity against MAGs (32, 33). None of these lipids were hydrolyzed by mutant Tgl2pS144A isolated from the mitochondria (data not shown).

Although it was completely inactive toward tripalmitin and dipalmitin, it has been reported that both pancreatic lipase and adipose tissue lipase hydrolyze DAGs better than TAGs but show little activity against MAGs (32, 33). None of these lipids were hydrolyzed by mutant Tgl2pS144A isolated from the mitochondria (data not shown). Taken together, our results imply that Tgl2p purified from the mitochondria possesses a strong lipase activity.

**TABLE 2**

| Lipid substrate | Specific activity nmol mg⁻¹ min⁻¹ |
|-----------------|----------------------------------|
| Triolein        | 13,000 ± 700                    |
| Dionein         | 14,000 ± 2000                   |
| Monolein        | ND                              |
| Tripalmitin     | ND                              |
| Dipalmitin      | ND                              |
| Monopalmitin    | ND                              |
| Tricaprylin     | 55,000 ± 5000                   |
| Dicaprylin      | 87,000 ± 3000                   |
| Monocaprylin    | ND                              |
| Tributyrin      | 70,000 ± 5000                   |
| Cardiolipin     | ND                              |
| Phosphatidylcholine | ND                           |

Although it was completely inactive toward tripalmitin and dipalmitin, it has been reported that both pancreatic lipase and adipose tissue lipase hydrolyze DAGs better than TAGs but show little activity against MAGs (32, 33). None of these lipids were hydrolyzed by mutant Tgl2pS144A isolated from the mitochondria (data not shown). Taken together, our results imply that Tgl2p purified from the mitochondria possesses a strong lipase activity.

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In a search for phenotypes associated with TGL2-deficient yeast, we found that the tgl2Δ mutant was more sensitive than the wild type to the anti-microtubule drug, benomyl (Fig. 6A). This phenotype was also confirmed by using another anti-microtubule drug, nocodazole (data not shown). As a control, tgl1Δ cells defective for sterol ester hydrolysis were also tested for the benomyl sensitivity. Tgl1p produces sterols and fatty acids by mobilizing sterol esters in the lipid particle (34). Similarly to the wild-type cells, tgl1Δ cells were not hypersensitive to benomyl (Fig. 6A). The benomyl-sensitive phenotype of the tgl2Δ mutation was rescued by expressing HA-TGL2 from the endogenous promoter. No suppression was observed by introducing the lipolysis-defective HA-TGL2S144A gene (Fig. 6B). Apparently, Tgl2 lipase is not required for the microtubule assembly or stability, because tgl2Δ cells exhibit little activity against MAGs (32, 33). None of these lipids were hydrolyzed by mutant Tgl2pS144A isolated from the mitochondria (data not shown). Taken together, our results imply that Tgl2p purified from the mitochondria possesses a strong lipase activity.

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We further examined whether benomyl sensitivity was suppressed by the addition of the fatty acid, the product of lipolysis,
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FIGURE 7. Biochemical interaction between Tgl2p and α-tubulin. Using rat monoclonal anti-yeast α-tubulin antibody (clone YOL1/34, Santa Cruz Biotechnology, Inc.) and protein G-Sepharose beads (Sigma), α-tubulin immune complex was prepared from tgl2Δ cells harboring pRS314–796-HA-TGL2 (lanes 1 and 3) or pYN04-HA-TGL2 (lanes 2 and 4), separated by SDS-PAGE, and immunoblotted for α-tubulin (lanes 1 and 2). The blot was washed and probed with anti-HA antibody (lanes 3 and 4). The enhanced chemiluminescent West Dura substrate (Pierce) was used to detect antigens. The positions of α-tubulin (solid circles) and HA-Tgl2p (arrowheads) are shown. The asterisks mark the position of the heavy chain from rat tgl2Δ.

FIGURE 6. Anti-microtubule drug sensitivity of the tgl2Δ mutant and phenotypic suppression by the lipolysis-competent TGL2 gene expression. A, 10-fold serial dilutions of tgl2Δ cells (tgl2Δ), tgl1Δ cells (tgl1Δ), and isogenic wild-type cells grown in YPD medium were spotted onto YPD plates containing DMSO (control), benomyl (85 μM), fatty acid (2 mM), or benomyl (85 μM) plus fatty acid (2 mM). Fatty acid added to the plate was butyric (C4:0), palmitoleic (C16:1), stearic (C18:0), and oleic (C18:1) acid. The assay was done as described in A but using synthetic glucose medium and plates lacking tryptophan to grow tgl2Δ cells transformed with pRS314 (No TGL2), pRS314-796-HA-TGL2 (HA-TGL2), or pRS314-796-HA-TGL2S144A (HA-TGL2S144A).

B, 10-fold serial dilutions of tgl2Δ cells (tgl2Δ) and tgl1Δ cells (tgl1Δ) in YPD plates containing DMSO (control) and benomyl (100 μM). Fatty acid added to the plate was butyric (C4:0), palmitoleic (C16:1), stearic (C18:0), and oleic (C18:1) acid. The assay was done as described in A but using synthetic glucose medium and plates lacking tryptophan to grow tgl2Δ cells transformed with pRS314 (No TGL2), pRS314-796-HA-TGL2 (HA-TGL2), or pRS314-796-HA-TGL2S144A (HA-TGL2S144A).

Of the four major fatty acids of yeast, palmitic (C16:0), palmitoleic (C16:1, Δ9), stearic (C18:0), and oleic (C18:1, Δ9) acid, only oleic acid fully complemented the benomyl-sensitive phenotype of TGL2- deficient yeast (Fig. 6A). Butyric acid (C4:0) was also tested because tributyrin served as the optimal substrate for Tgl2 lipase in vitro. It did not suppress the benomyl sensitivity of the tgl2Δ null mutation on the YPD plate (Fig. 6A). Additionally, caprylic acid (C8:0) failed to suppress the benomyl sensitivity (data not shown). Both wild-type and tgl2Δ cells grew equally well on medium containing each fatty acid in the absence of benomyl with the exception of palmitoleic acid (Fig. 6A). The addition of palmitoleic acid to the YPD medium was toxic to yeast cells. Of significance, oleic acid rescued the benomyl sensitivity of yeast cells defective for Tgl2p-dependent lipolysis in a concentration-dependent manner (Fig. 6B). The addition of 1 mM oleic acid was ineffective for suppression of drug sensitivity, whereas 2 mM oleic acid fully suppressed the defect. These data indicate that lipolytic activity of Tgl2p is required for its physiological role, and small epitope (HA) tagging does not interfere with this function. More specifically, our data imply that Tgl2 lipase improves the viability of benomyl-treated tgl2Δ cells via oleic acid production, and the limiting level of this unsaturated fatty acid is responsible for the detrimental consequence of perturbing microtubule assembly in these cells.

Notably, valuable information on the substrate specificity of Tgl2 lipase in vivo can be obtained from the present fatty acid suppression data, because the benomyl sensitivity of TGL2-deficient yeast reflects a defect in Tgl2p-dependent lipolysis. It is noteworthy that the major fatty acid distribution in cellular TAGs isolated from the triple mutant tgl3Δtgl4Δtgl5Δ exhibits an increase in C14:0, C16:0, C16:1, and C26:0 fatty acids but a decrease in C18:0 and C18:1 species (9). This suggests that besides Tgl3p, Tgl4p, and Tgl5p, other lipases capable of hydrolyzing C18:0 and C18:1 containing TAGs do exist in S. cerevisiae. It appears that Tgl2 lipase is highly specific toward a C18:1 unsaturated fatty acid, and this specificity is especially apparent in the absence of its overproduction, although the signal was very weak (Fig. 7, lanes 1 and 3).

Because deletion of the TGL2 gene conferred sensitivity to microtubule poisons, we performed immunoprecipitation-immunoblot analysis to see if Tgl2p interacted with yeast tubulin. HA-Tgl2p was found in α-tubulin immunoprecipitates even in the absence of its overproduction, although the signal was very weak (Fig. 7, lanes 1 and 3). However, both α-tubulin and HA-Tgl2p were clearly visible from precipitates of α-tubulin when Tgl2p was overproduced, confirming the interaction between Tgl2p and α-tubulin (Fig. 7, lanes 2 and 4). In the reverse experiment, α-tubulin was detected from precipitates of HA-Tgl2p only when the expression of TGL2 was elevated (data not shown). In brief, Tgl2p-dependent lipolysis or its
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likely product oleic acid seems to play an important role in the viability of cells under anti-microtubule drug treatment.

DISCUSSION

We present several lines of evidence that TGL2 encodes a functional lipase located in the S. cerevisiae mitochondria. First, HA-Tgl2p purified close to homogeneity from a tgl2Δ strain overproducing this fusion protein exhibits high TAG hydrolytic activity toward emulsified triolein. The S144A mutation in the lipase consensus motif (G/A)XSXG abolishes this activity. Second, deletion of TGL2 results in an increase in the cellular TAG content. Although overexpression of HA-TGL2 leads to an increase in TAG degradation in the presence of fatty acid synthesis inhibitor cerulenin, no significant change is observed by elevating the mutant HA-TGL2S144A gene expression. Finally, Tgl2p is recovered with pure mitochondria upon cellular fractionation, and Tgl2p-specific lipolytic activity is present in isolated mitochondria. Like serine 315 of Tgl4p, serine 144 of Tgl2p is essential for catalytic activity as evidenced by the loss of lipolytic activity both in vivo and in vitro when the residue is mutated to alanine. Mutant Tgl2pS144A having no catalytic activity is synthesized at levels comparable with the native protein and correctly localized to the mitochondria. In summary, we provide evidence that Tgl2p plays a direct role in yeast TAG mobilization for the first time.

Tgl2p is distinct from other yeast lipases with respect to subcellular localization. Previously, Schousboe (14, 15) has described a mitochondrial lipase activity from S. cerevisiae. This activity constituted ~46% of the total intracellular lipolytic activity and seemed to have no requirement for free Mg2+ or Ca2+. It appears that Tgl2p also has little requirement for free Mg2+ and Ca2+ as shown in Fig. 3D. The reported lipase activity of intact mitochondria was ~6.7 nmol mg-1 min-1 at a TAG concentration of 9.0 mm, pH 7.5, and 30 °C (15). The mitochondrial fraction prepared from cells expressing endogenous levels of TGL2 showed a specific activity of 0.4 ± 0.1 nmol mg-1 min-1 at pH 8 and 37 °C, using 3.6 mm triolein (Table 1). Thus, it is not clear at present whether Tgl2p is the sole lipase located in the mitochondria, although the activity in this organelle was undetectable in the absence of TGL2. Also, the two activities cannot be compared directly because the triolein concentration and other assay conditions are not identical. Nonetheless, Tgl2p possesses a strong lipase activity when purified from the mitochondria (Tables 1 and 2). To our knowledge, it is the first lipase identified in the yeast mitochondria.

An important clue to the physiological role of Tgl2p may be further provided by the current phenotypic analysis of the null mutation. The benomyl and nocodazole sensitivity of the TGL2-deficient yeast combined with physical interaction between Tgl2p and α-tubulin suggest that this protein may participate in the microtubule-mediated function (Figs. 6 and 7). Significantly, lipolysis catalyzed by Tgl2p is crucial for this role because in contrast to expression of the wild-type TGL2 gene, the benomyl sensitivity of the null mutation was not rescued by expression of the mutant TGL2S144A gene encoding inactive lipase (Fig. 6B). Anti-microtubule drugs like vinblastine and nocodazole specifically inhibit palmitoylation of tubulin, which has been implicated in hydrophobic interaction between microtubules and various intracellular membranes (35). However, because the addition of palmitate failed to complement the benomyl-sensitive phenotype of the tgl2Δ null mutation (Fig. 6), it is implausible that a probable decrease in the endogenous palmitate level, as expected from reduced TAG degradation in tgl2Δ cells, has made the deletion mutant hypersensitive to tubulin-targeting agents. In line with this, Tgl2p showed no lipolytic activity toward tripalmitin or dipalmitin in vitro (Table 2).

In S. cerevisiae, all effective microtubule poisons cause depolymerization of microtubules and mitotic arrest, and mutants defective in the process of nuclear migration or chromosome segregation are sensitive to these agents (36). Nuclear positioning and microtubule morphology in tgl2Δ cells are indistinguishable from those in wild-type cells, ruling out the involvement of Tgl2p in nuclear migration. According to our preliminary data, Tgl2p is not directly involved in the process of chromosome segregation because tgl2Δ cells are not blocked by benomyl or nocodazole at mitosis and segregate testate minichromosome normally.

Previously, it has been shown that S. cerevisiae cells with a mutation in the MDM2/OLE1 gene encoding the Δ9 fatty acid desaturase display defective mitochondrial movement and aberrant mitochondrial distribution (37). Not surprisingly, a product of the desaturase, oleic acid, performs an essential role in mitochondrial movement and inheritance (37). It is almost tempting to propose that Tgl2 lipase serves as a specific link between mitochondria and microtubules to establish proper localization of mitochondria in the cell. Unfortunately, movement and distribution of mitochondria inside the cell and during cell division require interaction with actin cytoskeleton and not microtubule-based structure in budding yeast (38). No appreciable effects on mitochondrial function or morphology are detected in the tgl2Δ mutant, also arguing against this role.

Alternatively, Tgl2 lipase may prevent death of cells arrested in mitosis by elevating the concentration of oleic acid in the mitochondria. We propose that lipolysis catalyzed by the mitochondrial Tgl2 lipase or its likely product oleic acid may become crucial for the survival of cells under stress-inducing conditions, including anti-microtubule drug treatment. Of course, Tgl2p-dependent lipolysis has no clear effects on budding yeast except for a modest increase in TAG content under normal conditions. TGL2-deficient yeast grows even better than the wild-type in the absence of stress. In support of our hypothesis, there are many reports suggesting a relationship between the distribution of fatty acids and some stresses. For instance, the composition of cellular fatty acids changes when S. cerevisiae cells are under acetaldehyde stress, showing an increase in C18:0 and C18:1 (oleic acid) levels and a decrease in C12:0, C14:0, C16:0, and C16:1 levels (39). In particular, the content of oleic acid is remarkably increased in these cells. Supplementation of oleic acid into the medium partially complements growth defects in acetaldehyde-sensitive mutants and ole1Δ cells, whereas it barely affects the growth rate of wild-type cells under the same stress (39). Future work on Tgl2p could uncover yet undefined function of TAG lipases. Further studies on the yeast lipases will be valuable in unravelling the molecular
basis of lipid metabolism in cell physiology and hopefully provide clues on the treatment of lipid-associated disorders.

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