Storage and degradation of triglycerides are essential processes to ensure energy homeostasis and availability of precursors for membrane lipid synthesis. Recent evidence suggests that an emerging class of enzymes containing a conserved patatin domain are centrally important players in lipid degradation. Here we describe the identification and characterization of a major triglyceride lipase of the adipose triglyceride lipase/Brummer family, Tgl4, in the yeast Saccharomyces cerevisiae. Elimination of Tgl4 in a tgl3 background led to fat yeast, rendering growing cells unable to degrade triglycerides. Tgl4 and Tgl3 lipases localized to lipid droplets, independent of each other. Serine 315 in the GXYSXG lipase active site consensus sequence of the patatin domain of Tgl4 is essential for catalytic activity. Mouse adipose triglyceride lipase (which also contains a patatin domain but is otherwise highly divergent in primary structure from any yeast protein) localized to lipid droplets when expressed in yeast, and significantly restored triglyceride breakdown in tgl4 mutants in vivo. Our data identify yeast Tgl4 as a functional ortholog of mammalian adipose triglyceride lipase.

Triglycerides (TG) serve different functions in a cell. First, they represent a most efficient way to store energy in the form of fatty acids (FA). Second, diglycerides (DG) liberated from TG by cleavage of a single fatty acyl ester bond, may serve as precursors for re-esterification to membrane phospholipids. Third, TG synthesis may also function as a sink to remove excess free fatty acids from the cellular milieu, in order to prevent FA-induced lipotoxicity. Because TG precursors or degradation products, such as phosphatidic acid or DG species, are also potential second messengers involved in multiple signaling processes, both TG synthesis and breakdown obviously require a stringent spatial and temporal control (1). Fueled by the epidemic dimensions of lipid-associated disorders, such as obesity and type 2 diabetes (2–4), numerous research strategies are focused toward understanding the genetic basis and molecular mechanisms that regulate uptake, synthesis, deposition, and mobilization of lipids, in the context of energy homeostasis (5–7). Because of the complexity of the problem, major input in this endeavor comes from the use of model systems, including mice, flies (Drosophila), worms (Caenorhabditis elegans) or yeast.

In yeast, mobilization of fat depots occurs as a consequence of at least three different metabolic stimuli: in stationary phase, upon nutrient depletion, fatty acids are released from TG depots rather slowly and are subjected to peroxisomal β-oxidation, providing the metabolic energy for cellular maintenance (8). Alternatively, lipid depots are degraded very rapidly in cells that exit starvation conditions, e.g. from the stationary phase, and enter a vegetative growth cycle upon supplementation with carbohydrates (9). Because peroxisomes are repressed under these conditions, storage lipid compounds including sterol esters (SE) and TG may rather be utilized for membrane lipid synthesis to support rapid initiation of cellular growth and division (10, 11). Lipolysis is essential also in the absence of cellular growth in diploid cells undergoing meiosis and sporulation. For instance, phospholipase D (Spo14) (12) or sterol ester hydrolase Tgl1 (13) are important for remodeling the lipid content for nuclear membrane and pro-sporule membrane formation, and the respective homozygous diploid mutants are unable to sporulate.

Obviously, exposure to different nutritional conditions requires the activity of multiple lipases to rapidly adjust cellular lipid pools. In mammalian cells, invertebrates, as well as in plants and fungi, including baker’s yeast, triglycerides and sterol esters are packaged into lipid droplets (LD) (14), which are “organelles” consisting of neutral fat surrounded by a phospholipid monolayer (15). In yeast, LD are believed to form by budding from the endoplasmic reticulum, which also harbors most of the enzymes required for sterol synthesis and esterification, and TG formation (16–18). Enzymes involved in TG and SE synthesis are remarkably conserved. The (re)acylation of diglyceride to triglyceride is carried out by two different enzymes in yeast, Dga1, which is an ortholog of mammalian DGAT2 (acyl-CoA:diacylglycerol acyltransferase) (19–22), and by Lro1, which is a phospholipid:diacylglycerol acyltransferase (PDAT). Yeast Lro1 is about 27% homologous to mammalian lecithin:cholesterol acyltransferase, LCAT (23, 24). Lro1 and Dga1 account for the majority of the yeast TG synthesis capacity, with some minor contribution also by Are1, the acyl-CoA:cholesterol acyltransferase (ACAT)-related enzyme, preferentially catalyzing sterol ester synthesis. Elimination of LRO1, DGA1, and ARE1 genes altogether renders yeast cells incapable of any detectable TG synthesis.

The recent discovery of adipose triglyceride lipase (ATGL) as the major lipase acting on TG in mouse adipocytes (Ref. 25; also termed desnutrin, Ref. 26) supports the notion that our understanding of the molecular mechanisms controlling TG homeostasis in the cell is incomplete. ATGL operates in conjunction with hormone-sensitive lipase (HSL), which was found to preferentially function as a diglyceride hydrolase (27). Monoglyceride lipase (MGL) catalyzes the final step in complete TG breakdown to free fatty acids and glycerol. Both enzymes, ATGL and HSL, act in concert to ensure efficient TG hydrolysis without DG accumulation, in adipocytes. Interestingly, ATGL contains a patatin domain (Pam01734), originally identified in patatin, the major protein of potato tubers (28), which displays esterolytic and phospholipase activity. Patatin lacks a typical catalytic triad, characteristic for most...
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lipases identified so far (29–34) and, instead, contains a catalytic diad, consisting of serine and aspartate residues, as deduced from its crystal structure (33). Although the primary sequence of the patatin domain is only poorly conserved, multiple proteins harboring this domain appear as an emerging class of enzymes in all types of eukaryotic cells, for most of which, however, a specific function has not been unveiled yet (35). Very recently the Drosophila ortholog of ATGL, Brummer lipase (encoded by bmm) was identified in a genome-wide screen for genes that are nutritionally regulated. Brummer localizes to lipid droplets, and fly embryos lacking both maternal or zygotic Brummer activity are inviable, demonstrating an essential function of this enzyme in flies (36).

Whereas enzymes involved in TG synthesis in yeast appear both structurally and functionally conserved to mammalian cells, the level of sequence conservation for TG-degrading enzymes is less pronounced. Although the recently characterized Tg11 protein of yeast displays more than 30% sequence identity to mammalian acid lipases, it may function as a steryl ester hydrolase rather than a TG lipase in vivo (13, 37). The only yeast TG lipase identified so far, Tg13 (38), lacks any significant structural homology to known lipases in higher eukaryotes. However, a functional ortholog of mammalian ATGL, and hence suggest the name patatin domain-containing proteins encode lipolytic enzymes in yeast Nte1 functions in yeast as an intracellular phospholipase B, involved in degradation of the mammalian neuropathy target esterase, NTE.

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Site-directed Mutagenesis of the Putative Serine Active Site —For construction of TGL4-S315G, site-directed mutagenesis was performed using primers, 5'-GGTACGCGGTGCTGGTGCAG-3' and 5'-GGCAGCGCGCTACCCTGACGCAGAGGAAACGGCGCAGGAATA-3' to convert the serine 315 coding ATG to GGT (glycine, underlined). The mutated gene was amplified and inserted into the pYES2-Plasmid, as described above, and sequenced.

Expression and Mutation of Murine ATGL under Control of the CUP1 Promoter in Yeast —For overexpression of GTPase under control of the yeast CUP1 promoter, murine ATGL was amplified using plasmid pcDNA4/HisMax-ATGL (25) with the primers 5'-CTAGTTAGATGACGAACTATCGATATGGACGAA-3' and 5'-GGCAGCGCGCTACCCTGACGCAGAGGAAACGGCGCAGGAATA-3' to yield murine ATGL cDNA under control of the yeast CUP1 promoter. Murine ATGL cDNA was cloned into the expression vector pYEX-4T-1 (Clontech).
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FIGURE 1. Kinetics of neutral lipid mobilization during growth in yeast. LD were visualized using the \( TGL4 \) promoter, and GFP fluorescence was excited with chloroform/methanol 2:1 (v/v) (46). Neutral lipid analysis was performed by desorption TLC essentially as described (16), by applying samples onto silica gel plates (Merck) with an automated sampler (Camag Automatic TLC Sampler 4). Total lipids were separated by using chloroform/methanol (2:1, v/v) as the solvent for triglyceride detection, or by using chloroform/methanol (2:1, v/v) as the solvent for triglyceride detection. Lipids were visualized on TLC plates by carbonization after dipping plates into 3.2% \( H_2SO_4 \) and 0.5% \( MnCl_2 \), followed by heating at 120 °C for 5 min. Lipids were quantified by densitometric scanning at 450 nm (Camag TLC scanner 3), using triolein as the standard.

Analysis of Triglyceride Degradation in Vivo—To analyze the degradation of triglycerides in vivo, yeast cells were broken with glass beads in a Merckenschlager homogenizer (B. Braun Biotech International) under \( CO_2 \) cooling, and lipids were extracted with chloroform/methanol, disintegrated by vigorous shaking with glass beads in a Merckenschlager homogenizer (B. Braun Biotech International) under \( CO_2 \) cooling, and further processed as described above.

Analysis of Triglyceride and Diglyceride Lipase Activity in Vitro—For determination of lipase activities in vitro, strain YCK1159, which is devoid of endogenous TG synthesis and degradation was transformed with plasmids harboring \( TGL3 \) or \( TGL4 \) reading frames under control of the \( GAL1/10 \) promoter, or the empty vector. After induction for 6 h in the presence of 2% galactose, enzyme extracts were prepared by homogenization of cells with glass beads under \( CO_2 \) cooling and two centrifugation steps at 1000 \( g \) to remove glass beads and unbroken cells. The triglyceride substrate was prepared as follows: 200,000 cpm of \( [3H] \) triolein (19.5 Ci/mmol) were dried under a stream of nitrogen, resuspended in 75 \( \mu \)l of 100 mM potassium phosphate buffer, pH 7.4, and sonicated for 4 min on ice (Virsonic; 475 watts at 100% output). 25 \( \mu \)l of defatted bovine serum albumin (20 mg/ml) were added. Diglyceride substrate was prepared as follows: 660,000 cpm of \( [3H] \) diolein (10.5 Ci/mmol) were dried under a stream of nitrogen, resuspended in 75 \( \mu \)l of 50 mM potassium phosphate buffer, pH 7.0 and 25 \( \mu \)l of 2.5% defatted bovine serum albumin. Cell homogenates (up to 400 \( \mu \)g of total protein) were added to 100 \( \mu \)l of prewarmed substrate solution (30 °C); after brief mixing, 100 \( \mu \)l were immediately withdrawn and extracted with 400 \( \mu \)l of chloroform/methanol (2:1, v/v). 100-\( \mu \)l aliquots of the incubation mixture were withdrawn after 15, 30, and 45 min of incubation at 30 °C, and extracted with 400 \( \mu \)l of chloroform/methanol for 1 h. The organic phase was removed, dried under a stream of nitrogen, and resuspended in 50 \( \mu \)l of chloroform/methanol (2:1, v/v). Lipids were separated by TLC using light petroleum/diethyl ether/acetate (70:30:2, per vol) as the solvent. Bands were visualized with iodine vapor and triglycerides, diglycerides, monoglycerides, and free fatty acids were scraped off the plate. The radioactivity was determined by liquid scintillation counting.

For measuring DG activity in vitro, 100 \( \mu \)l of substrate (3660,000 cpm) were incubated with cell homogenates (100 \( \mu \)g of protein) in a water bath at 37 °C for 60 min. The reaction was stopped after 1 h by the addition of 3.25 ml of methanol/chloroform/heptane (10:9:7, per vol) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, pH 10.5. After a centrifugation step (800 \( \times \) g, 15 min), the radioactivity in 1 ml of the aqueous phase was determined by liquid scintillation counting.

Microscopy—For localization studies, \( TGL3 \)-GFP-, \( TGL4 \)-GFP-, and \( mATGL \)-GFP-expressing cells were harvested after 12 h of growth in YPD or selective minimal medium, respectively, prior to microscopic inspection. To determine the localization of \( TGL4 \)-GFP hybrid proteins in \( tgl3 \) deletion strains and \( vice versa \), mutants were transformed with episcopal pUG35 plasmids harboring the \( TGL4 \)-GFP and \( TGL3 \)-GFP fusion genes under control of the \( MET25 \) promoter. Expression was induced on medium lacking methionine. GFP fluorescence was excited...
at 488 nm and detected in the range between 500 and 525 nm, on a Leica SP2 confocal microscope with spectral detection. Lipid droplets were co-stained with the hydrophobic dye Nile Red (47, 48) and fluorescence excited at 488 nm and detected in the range from 550 to 575 nm. This setup minimized fluorescence bleed-through of the GFP signal into the Nile Red detection channel, and avoided most of the red-shifted Nile Red fluorescence because of incorporation into membranous structures (48). Nomarski (differential interference contrast, DIC) optics was used to record transmission images.

Lipid droplet number and volume were calculated from serial sections of at least 100 cells expressing FAA4-GFP, at various stages of growth. Faa4 is a prototypic and fairly abundant lipid droplet marker, localizing to the surface of LD and the endoplasmic reticulum (17). Variability of LD volume estimated by quantitative image analysis was within 10% among at least three determinations.

Miscellaneous Methods—Transformation of yeast and E. coli cells was performed following standard procedures (49). Proteins were quantified by the method of Lowry et al. (50) using bovine serum albumin as the standard. For Western blotting, total cell extracts were precipitated with 10% trichloroacetic acid and then solubilized in 0.1% SDS, 0.1% NaOH, and separated by one-dimensional SDS-polyacrylamide gel electrophoresis (51) using 5% stacking and 10% separating gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad) and incubated overnight in blocking solution containing 5% bovine serum albumin in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0). After blotting, membranes were incubated for 1 h with mouse anti-His6 antibodies, washed three times in TBST, and incubated for 1 h with anti-mouse peroxidase-conjugated antibody. Peroxidase reaction was performed according to the manufacturer’s instructions (Amersham Biosciences), and detected on a Typhoon 9400 scanner. Sequence alignments were performed using the program Clustal X with the Blossum series; gap creation penalty 10; gap extension penalty 0.2; Blossum series). The bar indicates the conserved GXXG motif, characteristic for serine esterases.

RESULTS

Kinetics of Lipid Droplet Mobilization—Triglycerides are exclusively stored in lipid droplets in yeast. To characterize LD formation and degradation during growth, morphological changes of lipid droplets were visualized and quantitated, using the LD resident marker protein, Faa4 (fatty acid activation protein 4, Ref. 53) fused to green fluorescent pro-
tein. The Faa4-GFP fluorescence delineated more precisely the surface of the LD and also allowed optical separation of LD clusters, in contrast to Nile Red staining. Furthermore, we noticed that the efficiency of Nile Red staining was strongly dependent on the growth phase, yielding only low fluorescence signals in actively growing cells.

In stationary phase cells, Faa4 localized exclusively to LD (Fig. 1) and the LD volume, as determined by quantitative image analysis, was found to account for about 5% of the total cell volume. Upon transfer of stationary phase cells into fresh culture medium, LD volume dropped by 80% within the first 6–8 h of cultivation; during this lag phase, cells underwent one or two divisions, and Faa4 localization shifted toward the endoplasmic reticulum. Because 98% of the LD mass are composed of TG and SE, this drastic reduction in LD volume suggests strongly induced lipolytic activities during initiation of cellular growth. LD pools were replenished in growing cells, reaching highest levels as cells entered stationary phase (Fig. 1).

**Yeast Has Four Patatin Domain-containing Proteins**—In a computational search for potential lipases involved in TG and SE breakdown in growing cells, four proteins containing patatin domains (Pfam01734), namely Tgl3 (Ymr313c), Nte1 (Yml059c), Ykr089c, and Yor081c, were identified in the yeast proteome. These proteins contain GXSXG lipase motifs (29, 30, 34), but differ quite considerably with respect to size and primary structure. Tgl3 (73.6 kDa) was previously identified as a TG lipase in yeast (38), and Nte1 (187.1 kDa) is a homolog of the mammalian neuropathy target esterase I, NTE (39, 40), displaying intracellular phospholipase B activity. Fig. 2 shows the amino acid sequence alignment of the patatin domains of these yeast proteins, together with the Drosophila Brummer lipase and mouse ATGL. The level of sequence

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4 H. Wolinski and S. D. Kohlwein, unpublished observation.
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conservation beyond the GASXG lipase active site consensus sequence is rather low; however, the identification of two lipolytic activities among the four patatin domain proteins of the yeast proteome prompted us to investigate the function of YOR081c and YKR089c gene products (84.7 and 102.7 kDa, respectively) in greater detail: as a first step, deletion mutants were characterized with respect to their lipid composition and ability to degrade TG in vivo and in vitro.

Mutants Defective in Gene YKR089c Accumulate TG—Yeast deletion mutants lacking genes YOR081c or YKR089c were analyzed for their ability to mobilize lipids in vivo. yor081c mutants displayed wild-type levels of TG and SE, and degraded TG and SE at wild-type rates in vivo during growth, as determined by total lipid analysis (data not shown). In contrast, mutants lacking gene YKR089c accumulated TG up to 2-fold in early log phase (TG to phospholipid ratio is 1.0 mg/mg), compared with wild type (TG to phospholipid ratio is 0.5 mg/mg). Furthermore, ykr089c mutants showed significantly delayed TG degradation, comparable to tgl3 mutants, when stationary phase cells were supplemented with fresh growth medium (Fig. 3A). This reduced capacity to degrade TG for both tgl3 and ykr089c single mutants was even more pronounced under stringent conditions when de novo fatty acid synthesis was inhibited in the presence of 10 mg/liter cerulenin (Fig. 3B). tgl3 ykr089c double mutants were unable to hydrolyze TG under these conditions, suggesting that the respective gene products account for the majority of the cellular capacity to degrade TG. These observations were corroborated by staining of lipid droplets with Nile Red (Fig. 3C). Wild-type cells were virtually devoid of lipid droplets after cultivation for 6 h in the presence of cerulenin. In contrast, ykr089c single and tgl3 ykr089c double mutants retained significant Nile Red staining of neutral lipid depots after 6 h, indicative of a largely delayed mobilization of neutral lipids from the LD. These data suggest that the YKR089c gene product indeed functions as a TG lipase in vivo and was, hence, named Tgl4. Growth of tgl3 tgl4 double mutants was severely retarded (Fig. 3D), demonstrating that TG degradation is important for rapid initiation of growth.

Tgl4 Localizes to Lipid Droplets, Independent of Tgl3—To investigate the subcellular localization of both lipases, Tgl3 and Tgl4 were tagged with green fluorescent protein and analyzed by high resolution confocal laser scanning microscopy in living yeast cells. As shown in Fig. 4A, Tgl3-GFP and Tgl4-GFP localized exclusively to lipid droplets (indicated by co-localization with Nile Red) and were absent from the endoplasmic reticulum (Elo3-GFP was used as an ER marker, Ref. 43). Localization of Tgl4 protein was independent of the presence of Tgl3, and localization of Tgl3 to LD was also independent of the presence or absence of Tgl4. The exclusive localization of both Tgl3 and Tgl4 to LD and their absence from the ER is in contrast to most LD proteins identified so far, which show dual localization to these two organelles.

Tgl3 and Tgl4 Are Lipases with Different Substrate Preferences for DG and TG—To further substantiate their potential role as TG lipases we investigated the substrate specificity of Tgl3 and Tgl4 in vivo and in vitro. tgl3 tgl4 double mutants were transformed with plasmids harboring the single genes under control of the galactose-inducible GAL1/10 promoter. Overexpression of TGL4 in the double mutant resulted in 3-fold-stimulated degradation of TG and an accumulation of DG in vivo, during 6–7 h of induction. DG levels reached up to 12% of the total cellular lipid content, suggesting that Tgl4 preferentially degrades TG rather than DG (Fig. 5A). Overexpression of TGL3 also stimulated TG degradation, and led to DG accumulation up to 6% of total lipids, after induction. These data suggest that both Tgl3 and Tgl4 are lipases preferentially hydrolyzing TG. In addition, Tgl3 may have a higher affinity for degrading DG in vivo, compared with Tgl4.
mined by Western blotting, the specific activity of Tgl4 against TG is about 1.5-fold higher than that of Tgl3. White bars indicate the YCK1159 control strain transformed with the empty plasmid. Cells were grown for 16 h in minimal medium lacking uracil and with raffinose as the carbon source, prior to induction of the empty plasmid. Cells were grown for 16 h in minimal medium lacking uracil and with raffinose as sole carbon source. Cells were inoculated to medium containing galactose and supplemented with 10 mg/liter cerulenin, to induce TG mobilization. Lipids were extracted and analyzed at the indicated time points. B, Western blot analysis of His$_{6}$-tagged native Tgl4 and Tgl4-S315G proteins, confirming identical levels of expression in the transformed tgl4 mutant. Loading control; segment of the Coomassie Blue-stained gel demonstrating equal loading for the immunoblot.

and TG degradation in the presence of cerulenin was monitored in vivo, in comparison to mutant cells transformed with the TGL4 wild-type gene or with the control plasmid. Expression of the TGL4 wild-type gene resulted in stimulated TG hydrolysis, as expected (Fig. 6A). In contrast, expression of the mutated gene did not stimulate TG hydrolysis in the tgl4 mutant over the negative control. Similar expression levels of both the wild-type and mutated forms of Tgl4 were confirmed by Western blotting (Fig. 6B). These data show that serine 315 of the lipase active site consensus sequence in Tgl4 is essential for catalytic activity and support the notion of a lipolytic function of the enzyme, rather than a regulator of an as yet unknown lipase.

TG Mobilization Is Restored in Defective Yeast Mutants by Heterologous Expression of Murine ATGL—To test whether adipose triglyceride lipase from mouse can functionally replace endogenous yeast lipases encoded by TGL3 and TGL4, the mATGL gene was cloned and expressed in wild-type and tgl3 and tgl4 single or tgl3 tgl4 double mutant cells. First, we investigated the subcellular localization of heterologous mATGL. Remarkably, as shown in Fig. 4B, murine ATGL expressed as a C-terminal GFP fusion localized preferentially to lipid droplets in living yeast cells, and only about 10% resided in the cytosol, as quantitated by cell fractionation. mATGL expressed in tgl3 and tgl4 mutants also local-

FIGURE 5. Enzyme activities in vivo and in vitro. A, overexpression of TGL4 under control of a GAL1/10 promoter in tgl3 tgl4 double mutants resulted in the accumulation of diglyceride up to 12% of total cellular lipids (black bars). TGL3 overexpression in tgl3 tgl4 double mutants caused accumulation of DG up to 6% of total lipids (gray bar). Wild-type cells transformed with the empty plasmid as the control showed no detectable accumulation of diglyceride (white bar). Cells were grown for 16 h in minimal medium lacking uracil and with raffinose as the carbon source, prior to induction of GAL1/10-driven expression on 2% galactose. Lipids were extracted at the indicated time points and analyzed as described under “Materials and Methods.” B, analysis of DG lipase activity in vitro: cell extracts prepared from strain YCK1159 transformed with a TGL3 expression plasmid exhibited increased activity against DG as the substrate (gray bar); in contrast, extracts prepared from TGL4-expressing strains (black bar), did not show any detectable DG hydrolysis activity under these conditions. C, Tgl4 and Tgl3 displayed about equal lipolytic activity against triglyceride as the substrate, based on total protein in the cell extract. If the relative abundance of the two proteins is taken into account, as determined by Western blotting, the specific activity of Tgl4 against TG is about 1.5-fold higher than that of Tgl3. White bars indicate the YCK1159 control strain transformed with the empty plasmid. Cells were grown for 16 h in minimal medium lacking uracil and with raffinose as the carbon source, prior to induction of TGL3 and TGL4 gene expression on galactose, for 6 h. In vitro assays were performed using radiolabeled DG and TG substrates, as described under “Materials and Methods.” D, Western blot using anti-GST antibodies to show relative levels of expression for both Tgl4-GST and Tgl3-GST fusion proteins in cellular extracts, and the loading control segment of the Coomassie Blue-stained gel, demonstrating equal loading for the immunoblot.

FIGURE 6. Serine 315 is essential for lipolytic activity of Tgl4. The serine esterase consensus sequence GX$_{3}$XG$_{2}$ bearing the putative active serine residue was mutated by site-directed mutagenesis to GXGXXG. Mutation of serine 315 results in loss of Tgl4 activity. A, tgl4 mutants transformed with episomal wild-type TGL4 (■), TGL4-S315G (▲), and control plasmid (●) were pregrown for 42 h in minimal medium lacking uracil and with raffinose as sole carbon source. Cells were inoculated to medium containing galactose and supplemented with 10 mg/liter cerulenin, to induce TG mobilization. Lipids were extracted and analyzed at the indicated time points. B, Western blot analysis of His$_{6}$-tagged native Tgl4 and Tgl4-S315G proteins, confirming identical levels of expression in the transformed tgl4 mutant. Loading control; segment of the Coomassie Blue-stained gel demonstrating equal loading for the immunoblot.

TG Mobilization Is Restored in Defective Yeast Mutants by Heterologous Expression of Murine ATGL—To test whether adipose triglyceride lipase from mouse can functionally replace endogenous yeast lipases encoded by TGL3 and TGL4, the mATGL gene was cloned and expressed in wild-type and tgl3 and tgl4 single or tgl3 tgl4 double mutant cells. First, we investigated the subcellular localization of heterologous mATGL. Remarkably, as shown in Fig. 4B, murine ATGL expressed as a C-terminal GFP fusion localized preferentially to lipid droplets in living yeast cells, and only about 10% resided in the cytosol, as quantitated by cell fractionation. mATGL expressed in tgl3 and tgl4 mutants also local-

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ized to LD, demonstrating that its localization is independent of Tgl3 or Tgl4, respectively (not shown).

Next, degradation of TG was analyzed in vivo in yeast tgl3 and tgl4 single and tgl3 tgl4 double mutants transformed with mATGL, expressed under control of the GAL1/10 promoter. Lipid analysis unveiled that expression of murine ATGL stimulated TG breakdown in tgl4 mutants and in tgl3 tgl4 double mutants, but not in tgl3 mutants, despite the presence of about equal amounts of mATGL in these strains (Fig. 7). Given the substrate specificity of mATGL for triglycerides (25), these results are consistent with the in vivo and in vitro analyses obtained for Tgl3 and Tgl4. The localization of heterologous mATGL to yeast lipid droplets and the restoration of TG degradation in tgl4 mutants by mATGL strongly suggests that Tgl4 is indeed the yeast ortholog of mouse adipose triglyceride lipase and further demonstrates a remarkable level of functional conservation of lipolysis between yeast and mammals.

DISCUSSION

TG play a fundamental role in cellular metabolism. Accordingly, synthesis and mobilization of TG are regulated at multiple levels, dependent on nutritional and environmental signals. In the presence of nutrients, typically sugars, emphasis in yeast metabolism is directed toward cellular growth and proliferation. During that period of the yeast life cycle, TG accumulate and are stored in lipid droplets to sustain periods of starvation. Yeasts are able to endure extended periods of time, up to several weeks, in the absence of carbon sources (9). In this quiescent, non-proliferating state, cells presumably feed on fatty acids released from TG, providing energy for basic cellular functions. As sugars become available, β-oxidation of fatty acids is repressed. During that early phase of exposure to carbon source, TG may rather provide precursors for membrane lipid synthesis, to support rapid growth. Similarly, sterol esters are rapidly hydrolyzed under these conditions to release free ergosterol, which is essential e.g. for plasma membrane function. As cells enter vegetative growth and ample acetyl-CoA and ATP become available for highly energy-consuming fatty acid and sterol de novo syntheses, triglycerides and sterol ester pools are replenished.

Here we show that TG hydrolysis in yeast during early growth is catalyzed by two lipases, Tgl3 and Tgl4. Mutants lacking both enzymes display severely delayed growth, and reach only 50% of wild-type cell density, under identical nutritional conditions. Thus, rapid TG degradation during initial growth obviously provides a major advantage to the yeast cells. Remarkably, Faa1, a major acyl-CoA synthetase required for activation of free fatty acids (53), becomes essential for cells entering a new vegetative life cycle (54). Apparently, removal of free fatty acids that are liberated by lipase action and which requires activation with coenzyme A prior to incorporation into (membrane) lipids, is key for viability.

Both Tgl3 and Tgl4 are members of a growing family of TG lipases, including adipose triglyceride lipase/desnutrin (25, 26) and Brummer (36). These proteins are of highly divergent length and primary structure; however, they share a common yet poorly conserved patatin domain. The patatin domain contains the GXXG motif, which, in the case of Tgl4, was shown to be essential for catalytic activity. Interestingly, the protein encoded by gene YOR081c shares 55% homology to Tgl4, but does not appear to function as a TG lipase under normal growth conditions, and it does not overcome Tgl3 and/or Tgl4 deficiencies, in order to degrade TG in vivo. Because three other patatin domain-containing proteins in yeast harbor lipolytic functions, including the phospholipase B activity of the neu-

![FIGURE 7. Functional complementation of tgl4 and tgl4 tgl3 double mutants with adipose triglyceride lipase, mATGL from mouse.](image)

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Tgl4 and Tgl3 enzymes operate in parallel and independent of each other, and their localization to lipid droplets was constitutive and not affected by growth phase or nutritional conditions. Thus, regulation of lipolysis by translocation of the enzymes to the TG storage compartment appears unlikely. Recently, Tgl4 was identified as a substrate of cyclin-dependent protein kinase, Cdc28/CDK1 (55), suggesting that lipolysis in yeast may be regulated by CDK1-dependent phosphorylation of Tgl4.

The discovery of a high level of functional conservation of patatin domain-containing lipases between yeast and mammals, involved in a process highly relevant to human disease, opens new avenues for rapid functional testing of triglyceride lipolytic activities in a simple eukaryotic model system.

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