Screening for Hydrolytic Enzymes Reveals Ayr1p as a Novel Triacylglycerol Lipase in \textit{Saccharomyces cerevisiae}\textsuperscript{a,\textdagger}

Birgit Ploier\textsuperscript{1,}, Melanie Scharwey\textsuperscript{f,}, Barbara Koch\textsuperscript{1,}, Claudia Schmidt\textsuperscript{e,}, Jessica Schatte\textsuperscript{1,}, Gerald Rechberger\textsuperscript{v}, Manfred Kollroser\textsuperscript{s}, Albin Hermetter\textsuperscript{e}, and Günther Daum \textsuperscript{\textdagger}

From the \textsuperscript{1}Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria, the \textsuperscript{f}Institute of Molecular Biosciences, University of Graz, Humboldtstrasse 50, A-8010 Graz, Austria, and the \textsuperscript{e}Institute of Forensic Medicine, Medical University of Graz, Universitätsplatz 4/II, A-8010 Graz, Austria

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\textbf{Background:} Triacylglycerols are stored in lipid droplets and can be mobilized by lipases.

\textbf{Results:} Ayr1p was identified as a novel triacylglycerol lipase.

\textbf{Conclusion:} In addition to the known lipases Tgl3p, Tgl4p, and Tgl5p, further hydrolytic enzymes contribute to the mobilization of non-polar lipids in yeast.

\textbf{Significance:} This study opens the view for a broader network of lipolytic enzymes in yeast.

\textit{Saccharomyces cerevisiae}, as well as other eukaryotes, preserves fatty acids and sterols in a biologically inert form, as triacylglycerols and steryl esters. The major triacylglycerol lipases of the yeast \textit{S. cerevisiae} identified so far are Tgl3p, Tgl4p, and Tgl5p (Athenstaedt, K., and Daum, G. (2003) \textit{YMR313c/TGL3} encodes a novel triacylglycerol lipase located in lipid particles of \textit{Saccharomyces cerevisiae}. \textit{J. Biol. Chem.} 278, 23317–23323; Athenstaedt, K., and Daum, G. (2005) Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast \textit{Saccharomyces cerevisiae}, are localized to lipid particles. \textit{J. Biol. Chem.} 280, 37301–37309). We observed that upon cultivation on oleic acid, triacylglycerol mobilization did not come to a halt in a yeast strain deficient in all currently known triacylglycerol lipases, indicating the presence of additional not yet characterized lipases/esterases. Functional proteome analysis using lipase and esterase inhibitors revealed a subset of candidate genes for yet unknown hydrolytic enzymes on peroxisomes and lipid droplets. Based on the conserved GXSGX lipase motif, putative functions, and subcellular localizations, a selected number of candidates were characterized by enzyme assays \textit{in vitro}, gene expression analysis, non-polar lipid analysis, and \textit{in vivo} triacylglycerol mobilization assays. These investigations led to the identification of Ayr1p as a novel triacylglycerol lipase of yeast lipid droplets and confirmed the hydrolytic potential of the peroxisomal Lpx1p \textit{in vivo}. Based on these results, we discuss a possible link between lipid storage, lipid mobilization, and peroxisomal utilization of fatty acids as a carbon source.

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\textsuperscript{\textdagger} This article contains supplemental Table 1.
\textsuperscript{f} To whom correspondence should be addressed: Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, A-8010 Graz, Austria. Tel.: 43-316-873-6462; Fax: 43-316-873-6952; E-mail: guenther.dau@ tugraz.at.

In \textit{Saccharomyces cerevisiae} as well as in other eukaryotes, an excess of fatty acids is stored as triacylglycerols (TG)\textsuperscript{2} and steryl esters (SE), often referred to as non-polar lipids. Both lipids are stored in organelle-like structures called lipid droplets (LD), which are about 400 nm in diameter and consist of a highly hydrophobic core of TG, surrounded by shells of SE and a phospholipid monolayer containing a distinct set of proteins (1–4). TG are synthesized by the acyltransferases Dga1p and Lro1p, and SE are synthesized by the SE synthases Are1p and Are2p (5–10). All TG- and SE-synthesizing enzymes are located in the endoplasmic reticulum, but Dga1p is also found on LD. TG serves as the main energy storage, and both TG and SE are depots of membrane lipid components. Upon requirement (i.e. during growth or starvation), TG and SE can be mobilized by lipases or hydrolases. Currently, three major TG lipases are known, namely Tgl3p, Tgl4p, and Tgl5p, which are located on LD (11, 12). The hydrolysis of SE is conducted by Tgl1p and Yeh1p localized to LD and Yeh2p, which was found to be associated with the plasma membrane (13–16).

Tgl3p, Tgl4p, and Tgl5p share a common consensus sequence GXSGX, where serine is the essential residue of the catalytic triad aspartic acid/glutamic acid and histidine (17). They also contain a patatin domain, named after a plant storage protein that possesses lipid acylhydrolase activity (18). \textit{In vitro}, all three proteins exhibit lipolytic activity, whereas \textit{in vivo} only Tgl3p and Tgl4p mobilize TG efficiently. Previous studies from our laboratory have described functions for Tgl3p, Tgl4p, and Tgl5p in addition to their lipase activities. Tgl3p, Tgl4p, and Tgl5p harbor an acyltransferase motif (HX_{4}D), and \textit{in vitro} enzyme assays showed that both Tgl3p and Tgl5p act as lysophospholipid acyltransferases. Besides the conserved lipase motif, Tgl4p contains a (G/A)XGSGX calcium-independent

\textsuperscript{2} The abbreviations used are: TG, triacylglycerol(s); LD, lipid droplet(s); Px, peroxisome(s); QM, quintuple mutant; SE, steryl ester(s); TM, triple mutant; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-Nβ-sn-1/3-TGP, nitrobenz-2-oxa-1,3-diazole-snl/3-triacylglyceride phosphonate; NBD-HE-HP, O-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)hexanoyl)-aminophenyl-\textit{P}-(n-hexyl)phosphonic acid \textit{P}-nitrophenyl ester; pNPB, \textit{P}-nitrophenylacetate; pNPA, \textit{P}-nitrophenylacetate.
Yeast Lipase

TABLE 1
Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| WT     | BY4741 Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0 | Euroscarf |
| TM     | WT; tgl3Δ::kanMX4; tgl4Δ::kanMX4; tgl5Δ::kanMX4 | Ref. 12 |
| QIM    | tgl3Δ::kanMX4; tgl4Δ::kanMX4; tgl5Δ::kanMX4; Kindly provided by A. Wagner |
| LPX1+  | TM + pYEX4T-1-LPX1 | This study |
| LDH1+  | TM + pYEX4T-1-LDH1 | This study |
| YL13+  | TM + pYEX4T-1-YL13 | This study |
| AYR1+  | TM + pYEX4T-1-AYR1 | This study |
| EHT1+  | TM + pYEX4T-1-EHT1 | This study |
| TSC10+ | TM + pYEX4T-1-TSC10 | This study |
| YBR056w+ | TM + pYEX4T-1-YBR056w | This study |
| YKL050c+ | TM + pYEX4T-1-YKL050c | This study |
| AYR1S18A+ | TM + pYEX4T-1-AYR1S18A | This study |
| VC     | TM + pYEX4T-1 (vector control) | This study |

phospholipase A$_2$ domain. Phospholipase as well as SE hydrolase activity of Tgl4p was also established in vitro (19). AΔtgl3Δtgl4Δtgl5 (TM) yeast strain lacking all three TG lipases does not reveal any growth defect under standard growth conditions, although mutations in TGL3 or TGL4 lead to fat yeast cells that accumulate TG (19–21). Interestingly, we observed that upon cultivation on oleic acid, TG mobilization did not come to a halt in the TM deficient in all currently known TG lipases, suggesting the presence of novel yet not characterized hydrolases.

*S. cerevisiae* grown in the presence of oleic acid proliferates peroxisomes (Px) and at the same time accumulates large LD (22). Px are small ubiquitous organelles involved in the decomposition of toxic substances like H$_2$O$_2$ as well as degradation of fatty acids via β-oxidation. The mechanism of fatty acid transport to its site of degradation is not yet completely understood. In contrast to mammalian cells, the degradation of fatty acids in the yeast exclusively takes place in Px (23–25). Thus, functional Px are crucial for growth of yeast cells on fatty acids as a carbon source. Binns et al. (26) proposed a direct link between Px and LD, indicating a putative pathway for lipid supply to Px. It was suggested that Px can even penetrate LD, forming a structure called pexopodia, and that this contact may stimulate non-polar lipid turnover.

The aim of the present study was to identify novel hydrolytic enzymes possibly involved in non-polar lipid metabolism in the yeast *S. cerevisiae*. Our data suggest that in addition to Tgl3p, Tgl4p, and Tgl5p, further hydrolases are responsible for the mobilization of TG. Our approach identified a novel TG lipase and shed more light on a possible link between lipid storage, lipid mobilization, and peroxisomal utilization of fatty acids as a carbon source.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Table 1 gives an overview of strains used in this study. Yeast cells were either grown in YPD medium containing 1% yeast extract, 2% glucose, and 2% peptone or on oleic acid-supplemented medium (YPD) containing 0.3% yeast extract, 0.5% peptone, 0.1% glucose, 0.5% KH$_2$PO$_4$, and 0.1% oleic acid. For solubilizing oleic acid in YPO, 0.2% Tween 80 was added to the medium. Yeast strains bearing plasmids were cultivated in synthetic minimal medium (SD) containing 0.67% yeast nitrogen base (U. S. Biochemical Corp.), 2% glucose, and the respective amino acid supplements. If not stated otherwise, all cells were cultivated in liquid media at 30°C under vigorous shaking until early stationary phase. For gene expression studies, yeast cells were grown to the mid-exponential phase. Growth was monitored by measuring optical density at 600 nm ($A_{600}$). Expression of *CUP1* promoter-controlled genes was induced after growth for 12 h by adding CuSO$_4$ at a final concentration of 0.5 mM to the medium.

**Genetic Techniques**—A list of primers used for creating deletion cassettes is shown in Table 2. Gene deletions were performed following the PCR-mediated method described by Longtine et al. (27). Deletion cassettes were transforming the high efficiency lithium acetate transformation protocol (28). Correct integration of the knock-out cassettes was verified by growth auxotrophy as well as colony PCR.

For the expression of candidate hydrolases/lipases, the open reading frames of the respective genes were amplified from BY4741 chromosomal DNA using primers listed in Table 2. Restricted PCR fragments of *LPX1*, *LDH1*, *YL13*, *AYR1*, and *YBR056w* were inserted into the BamHI and EcoRI sites, whereas *EHT1*, *TSC10*, and *YKL050c* were inserted into the BamHI and SalI sites of the plasmid pYEX4T-1, a vector for high level expression of glutathione S-transferase (GST) fusion proteins in yeast under the control of a copper-inducible *CUP1* promoter.

Point mutations in *AYR1* were introduced by site-directed mutagenesis. Plasmid pYEX 4T-1_AYR1S18A was constructed by overlap extension PCR using the primers listed in Table 2.

**Isolation of Organelles**—Isolation of highly purified LD and Px was performed as previously described (29–31) and will only be described in brief here. For the preparation of Px, cells were grown in YPO to the late exponential phase. After harvesting, washing, and determining of the cell wet weight, cells were incubated with 0.5 g/ml SP-A (0.1 M Tris/SO$_4$, pH 9.4) and 1.54 mg of DTT/ml of SP-A for at least 10 min at 30°C with shaking. Cells were then washed and resuspended in prewarmed SP-B (1.2 M sorbitol, 20 mM KH$_2$PO$_4$, pH 7.4), and spheroplasts were generated by using Zymolyase-20 T (Seikagaku Corp.) at a concentration of 2 mg/g cell wet weight in 6 ml of SP-B/g of cell wet weight for 1 h at 30°C with shaking. The resulting spheroplasts were then washed with cold SP-B and resuspended in breaking buffer (5 mM MES/KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, and 0.5 mM EDTA) in twice the cell volume. The cell suspension was homogenized on ice using a Dounce homogenizer with a tight fitting pestle. Nuclei, unbroken cells, and cell debris were removed by centrifugation at 5,000 × g for 5 min in a Sorvall SLC3000 rotor. The resulting pellet was resuspended twice, rehomogenized, and centrifuged again to enhance the yield. The combined supernatants were centrifuged at 15,000 × g for 15 min in an SS34 rotor. The organelle pellet, consisting of mitochondria and Px, was again suspended in breaking buffer and centrifuged at 5,000 × g for 5 min to clean it from larger aggregates. The centrifugation step at 15,000 × g was repeated, and after suspension of the pellet in breaking buffer, it was loaded onto a Nycodenz gradient (17–35%; w/v) in 5 mM MES/KOH, pH 6.0, 1 mM KCl, and 0.24 M sucrose. The loaded gradient tubes were then centrifuged at 26,000 × g for 90 min in a swing out rotor (Sorvall AH-629). The white Px layer was taken with a
syringe, diluted with 4 volumes of breaking buffer, and sedimented at 15,000 × g in an SS34 rotor for 15 min at 4 °C.

For the isolation of LD, cells were grown in minimal medium (SD) to the early stationary phase. After preparation of spheroplasts as described above, they were resuspended in 1 ml/g cell wet weight LD-A (12% Ficoll 400 in 10 mM MES/Tris, pH 6.9, 0.2 mM Na₂EDTA·2H₂O), followed by mechanical disintegration using a Dounce homogenizer with a loose fitting pestle. The resulting homogenate was diluted with a half-volume of LD-A and centrifuged at 7,000 × g for 5 min at 4 °C. The supernatant was collected, and the pellet was resuspended in LD-A. Spheroplast disintegration and centrifugation were repeated, and the combined supernatants were transferred into Ultra-Clear centrifuge tubes (Beckman), up to one-third of height for 30 min at 4 °C. The supernatant was further used for protein determination and lipid extraction pending the cell pellet in breaking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and disintegrating by vigorous shaking in the presence of glass beads for 10 min at 4 °C. After disruption, cell debris were removed at 5,000 × g for 5 min. The supernatant was further used for protein determination and lipid extraction.

**TABLE 2**

| Primers used for construction of deletion mutants | Sequence 5’ → 3’ |
|-----------------------------------------------|----------------|
| Delare1fw                                      | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Delare1rev                                    | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Delare2fw                                      | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Delare2rev                                    | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Are1fwctrl                                    | GAAAAATAATGAGATGGTGAAGTGT |
| Are1revctrl                                   | ATGCTTGGCGACCAGATTTACC |
| Are2fwctrl                                    | CTTCATCAATGCAATCTATATTTCG |
| Are2revctrl                                   | GAATGCTTGGATGTGGTATCAT |

**Primers used for construction of overexpressing constructs**

| Name | Sequence 5’ → 3’ |
|------|----------------|
| Lpx1fw | GCGGATCTTCTAGATTGTTAGTCTGGATTTAAACC |
| Lpx1rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Ldh1fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Ldh1rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Yju3fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Yju3rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Ay1fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Ay1rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Eht1fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Eht1rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Tsc1fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Tsc1rev | CGGGATTCTTATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| YBR056fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| YBR056rev | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| YKL050fw | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| YKL050rev | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |

**Primers for site-directed mutagenesis**

| Name          | Sequence 5’ → 3’ |
|---------------|----------------|
| Are1_S18Afw   | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |
| Are1_S18Arev  | GTCGAGATTCATGGAACAGAAGGCTCGAAGAAAGTCAAGAAGG |

**Protein Determination**—The protein concentration of isolated fractions and whole cell extracts was determined by the method of Lowry et al. (32) using bovine serum albumin as a standard. Samples of LD fractions were delipidated with 2–3 volumes of diethyl ether prior to protein analysis. 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. Purified proteins were quantified by using the Bio-Rad protein assay based on the method of Bradford (33) and bovine serum albumin as a standard. 10 μg of each fraction were loaded onto SDS gels for Western blot analysis. SDS-PAGE was performed by the method of Laemmli (34) using 12.5% separation gels. Proteins on gels were detected by staining with Coomassie Blue. Western blot analysis was performed according to Haid and Sussa (35). Samples were denatured at 37 °C to avoid hydrolysis of polypeptides. Proteins were detected by a primary antibody directed against the GST tag and a peroxidase-conjugated secondary antibody. Enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce) were used to visualize immunoreactive bands.

**Lipid Analysis**—For lipid analysis, crude homogenates were prepared by harvesting cells in the early stationary phase, resuspending the cell pellet in breaking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and disintegrating by vigorous shaking in the presence of glass beads for 10 min at 4 °C. After disruption, cell debris were removed at 5,000 × g for 5 min. The supernatant was highly purified LD. The top layer was collected with a pipette and transferred into a 7-ml Dounce homogenizer, and LD were homogenized for further analysis.

Enhanced chemiluminescent signal detection reagents (SuperSignal™, Pierce) were used to visualize immunoreactive bands.

**Yeast Lipase**

For lipid analysis, crude homogenates were prepared by harvesting cells in the early stationary phase, resuspending the cell pellet in breaking buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and disintegrating by vigorous shaking in the presence of glass beads for 10 min at 4 °C. After disruption, cell debris were removed at 5,000 × g for 5 min. The supernatant was highly purified LD. The top layer was collected with a pipette and transferred into a 7-ml Dounce homogenizer, and LD were homogenized for further analysis.

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Yeasts Lipase

by the method of Folch et al. (36), using chloroform/methanol (2:1; v/v) as solvent. For the quantification of non-polar lipids, extracts were applied to Silica Gel 60 plates, and chromatograms were developed by a two-step developing system using first the solvent system light petroleum/diethyl ether/acetic acid (35:15:1) for two-thirds of the plate. Plates were dried briefly and further developed to the top of the plate using the second solvent system light petroleum/diethyl ether (49:1; v/v). Non-polar lipids were visualized by postchromatographic charring after dipping the plates in 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. The plates were then heated at 105 °C for 40 min. Visualized bands were quantified by densitometric scanning (CAMAG TLC SCANNER 3) at 400 nm with authentic standards containing defined amounts of the respective lipids.

Radioactive in Vivo TG Mobilization Assay—Precultures from wild type BY4741 (WT) and tgl3Δtgl4Δtgl5Δare1Δare2Δ (QIM) were used to inoculate a main culture to an A₆₀₀ of 0.1 in 100 ml of liquid YPO containing 10 μl of [³H]oleic acid (50 μCi = 110,000,000 dpm). Cells were grown for 20 h at 30 °C with shaking. Then cells were harvested and washed twice with a sterile solution containing 0.5% bovine serum albumin (essentially fatty acid-free; Sigma) and resuspended in 100 ml of non-labeled YPO. Cells were incubated at 30 °C, and aliquots were taken at the time points indicated. Cells were washed with 0.5% bovine serum albumin prior to cell density measurement at 600 nm. Aliquots of 10 ml were harvested at 4,500 × g for 5 min in a Hettich table top centrifuge. Cells were washed with 0.5% bovine serum albumin, and cell pellets were frozen at −20 °C and then used for lipid extraction as described above. Dried lipid extracts were separated by TLC as described above. Bands of TG were scraped off and, after the addition of 7 ml of scintillation mixture, subjected to scintillation counting using LSC of TG were scraped off and, after the addition of 7 ml of scintillation mixture, subjected to scintillation counting using LSC.

Table 3

| Primer | Sequence (5′ → 3′) |
|--------|------------------|
| ACT1_RTfw | CGACCTGTCTCCAGCCTGCAACAGAC |
| ACT1_RTrev | GATGTCATATCCGCAGTTATGAGCTT |
| LPX1_RTfw | AATTCTAGCTTTTAAAGAGGCTGCAATTTCC |
| LPX1_RTrev | CAGATTGCGTCGCGCACATGTTG |
| LDH1_RTfw | CTCAAGATGCGCTTATGGAATCCAGG |
| LDH1_RTrev | GCTCGAACAAACTCGCTTGTGACC |
| YJU3_RTfw | AGGAACTCCATTGAGCTTGAAGTCC |
| YJU3_RTrev | TCTGTATAATTGCCTTAACGACCA |
| AYR1_RTfw | GTGCTATAGGCGAGCGCCCCGGTGAG |
| AYR1_RTrev | CACGTGCGTGGCTTTGTCCTTAC |
| EHT1_RTfw | TCTAGAGCTCCCCTCGCGACCA |
| EHT1_RTrev | TCCCCTCGGTATTAAAGCTCTTTG |
| TSC10_RTfw | CAAGCTAGTGCACAGAGTGTAAG |
| TSC10_RTrev | ACGCGCTTCTTCTGCCGGGAC |
| YBR056w_RTfw | GGGACTTGGCGTTTGGATCGDG |
| YBR056w_RTrev | AAGCTCATCCAGCCGGAAG |
| YK1050c_RTfw | GCTGTTTGGATGAGCGCCCTAA |
| YK1050c_RTrev | CCGGGTTCCACATTGCTTGCG |

Birner-Gruenberger et al. (41). In addition, a detailed description of the method is provided in the supplemental material. Protein quantification was carried out as described above.

Protein Expression, Solubilization, and Purification—All candidate proteins were expressed from plasmid pYEX4T-1 in S. cerevisiae TM. GST fusion proteins were purified batch-wise by affinity chromatography using reduced glutathione agarose. Ayr1p and Eht1p were purified from LD fractions after solubilization by Zwittergent® (Sigma) at a final concentration of 20 mM; Lpx1p was solubilized from Px fractions with Zwittergent® at 10 mM; Ldh1p and YBR056wp were obtained from homogenates after solubilization with 2 mM Zwittergent. Reduced glutathione-agarose beads (Invitrogen) were washed with PBS to remove azide and equilibrated with PBS containing 1% Triton X-100 at 4 °C just prior to use. Solubilized fractions were applied to GST-beads and incubated for 2 h with moderate shaking at 4 °C. After washing with PBS, fusion proteins were eluted with 20 mM reduced glutathione (Sigma) in PBS, pH 8, at 4 °C for 30 min.

Esterase Activity Assays Using p-Nitrophenyl Esters as Substrates—p-Nitrophenylacetate (pNPA), p-nitrophenylbutyrate (pNPB), and p-nitrophenylpalmitate were used as substrates to determine esterase activity of purified proteins. The total reaction volume was 1 ml, containing 890 μl of 1× PBS buffer, 100 μl of substrate solution (ranging from 25 μM to 10 mM final concentrations), and 10 μl of purified protein, which was added directly prior to measurement. Absorbance was measured at 405 nm for 3 min at 30 °C. Enzyme activity was determined using different substrate concentrations. Michaelis-Menten kinetics was analyzed using GraphPad Prism version 5.

RNA Isolation and Real-time PCR—For the isolation of RNA, cells were grown to the midlogarithmic phase on YPD and YPO at 30 °C. RNA was isolated using the RNeasy kit from Qiagen as described by the manufacturer. After Dnase I digestion, real-time PCR was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) by following the manufacturer’s instructions. 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 (42). Differences in mRNA expression after ACT1 normalization relative to the control can be calculated with this method. Primers used for real-time PCR are listed in Table 3.

In Vivo TG Mobilization Assays—TG mobilization of strains overexpressing putative lipases/esterases was measured in vivo by letting cells grow until the early stationary phase in synthetic minimal medium. Then fresh medium was inoculated with the pregrown cultures to an A600 of 3. For the mobilization of TG, the fatty acid synthesis inhibitor cerulenin was added to a final concentration of 10 μM/ml. At the time points indicated, 10-ml aliquots were taken, cells were harvested, and lipids were extracted and analyzed as described above.

Triacylglycerol Lipase Activity Assays Using [9,10-3H]Triolein as Substrate—TG lipase activity of isolated LD and purified enzymes was determined using [9,10-3H]triolein (PerkinElmer Life Sciences) as substrate. TG 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, 125 μM [9,10-3H]triolein (specific activity 50 Ci/mmol), 45 μM phosphatidylcholine/phosphatidylinositol (3:1; mol/mol), 25 mM MgCl₂, and 0.2% fatty acid-free bovine serum albumin at 30 °C for 1 h in a water bath. The reaction was stopped by adding 2 ml of chloroform/methanol (2:1, v/v), and lipids were extracted as described above. Lipids were dried under a stream of nitrogen, dissolved in 50 μl of chloroform/methanol (2:1, v/v), and separated by TLC. Chromatograms were developed using chloroform/acetone/acetic acid (45:4:0.4, v/v/v) as a solvent system. Lipids were visualized by staining with iodine vapor, and bands corresponding to fatty acids were scraped off the plate. Radioactivity was measured by liquid scintillation counting using LSC Safety (Baker) with 5% water as a scintillation mixture. Enzyme activity was determined, depending on the triolein substrate concentration (0.2–250 μM) and time (1–200 min). Michaelis-Menten kinetics was analyzed using GraphPad Prism version 5.

RESULTS

A Yeast Strain Lacking All Currently Known TG Lipases Is Able to Mobilize TG in Vivo—The mobilization of TG from LD requires the catalytic activities of TG lipases. Previous research from our laboratory identified Tgl3p as the major TG lipase and the two enzymes Tgl4p and Tgl5p as showing minor lipolytic activities (11, 12). In the course of these studies, however, it became evident that the turnover of TG in TM did not come to a complete halt when cells were forced to mobilize TG in the presence of cerulenin, an inhibitor of fatty acid synthesis, suggesting the presence of additional TG lipases. To test this hypothesis, we constructed a mutant strain lacking both acyl-CoA:sterol acyltransferases, Are1p and Are2p, as well as the major known TG lipases Tgl3p, Tgl4p, and Tgl5p and performed a pulse-chase assay monitoring the fate of TG in vivo. This QiM accumulated high levels of TG but did not produce any SE, ensuring that incorporation of [3H]oleate occurred primarily into TG and that the loss of label due to incorporation into other compounds was minimized. Cells were pulse-labeled with [3H]oleic acid, and levels of 3H in TG were followed during the chase on unlabeled YPO. Both wild type and QiM grew normally under the given conditions (Fig. 1A). As expected, in wild type cells, the label in TG decreased over the time, indicating that TG was subject to turnover. Interestingly, in the QiM, lacking all known TG lipases, TG mobilization was observed with a slight delay but at a similar rate as wild type (Fig. 1B). Thus, we concluded that in cells grown on oleic acid, one or more additional lipases might become active, accounting for TG mobilization.

Screening for Novel Lipases and Hydrolases in LD and Px—The findings described above led us to search for novel TG lipases/hydrolases in the yeast. To test whether such enzymes became active in yeast grown on oleic acid, we employed a functional proteome assay making use of fluorescently labeled inhibitors of serine lipid hydrolases as probes (37, 38). Isolated organelles from the yeast were used to screen for such novel enzymes. LD from cells grown on glucose or oleic acid, respectively, as well as Px from YPO-grown cells were incubated with fluorescent phosphonic acid esters (NBD-sn1/3-TGP and NBD-HE-HP), which covalently bind to the active sites of serine lipid hydrolases. After incubation, proteins were separated by SDS-PAGE, and labeled polypeptides were identified by the NBD-reporter tag. In LD from wild type grown on YPD and YPO, protein bands of the size of the three lipases Tgl3p (~85 kDa), Tgl4p (~102 kDa), and Tgl5p (~85 kDa) were detected (Fig. 2A, asterisks). Moreover, bands representing possible new lipases were identified as well (Fig. 2A, arrows). A comparison of the two growth conditions revealed that the protein pattern of cells grown on YPO was different from YPD. We concluded from these data that indeed a different subset of proteins existed when cells were grown in the presence of oleic acid compared with glucose. To follow up this idea, we analyzed LD
from a TM grown on YPD or YPO, respectively. Again, we were able to detect additional protein bands that did not correspond to the already known TG lipases (Fig. 2B, arrows).

Similar results were obtained when we analyzed Px from wild type and TM with the same subset of inhibitors (Fig. 2C). Arrows indicate the occurrence of novel proteins exhibiting an esterase (NBD-HE-HP) or even lipase (NBD-sn1/3-TGP) activity. Proteins detected were of different molecular mass compared with those from LD. Thus, we speculated that on LD and Px, distinct hydrolases were expressed under oleic acid-induced conditions. Altogether, our screening apparently identified new and not yet characterized candidates for novel hydrolases/lipases on LD and Px. To identify these proteins reacting with NBD-HE-HP, they were separated by SDS-PAGE (Fig. 3A), tryptically digested, and subjected to MS analysis. A complete list of proteins identified by this approach is shown in supplemental Table 1. Proteins are listed according to their respective molecular weight as well as by peptide length and score value. All known TG lipases were identified with high abundance in LD samples. One exception was Tgl5p, which was found only on LD from cells grown on YPD. Interestingly, only a few gene products were detected in this screening in more than one organelle sample (see supplemental Table 1).

As expected, both LD samples showed the highest overlap in protein candidates (Fig. 3B). Eighteen candidate proteins were found on LD from cells grown on both carbon sources. These proteins represented putative new candidate hydrolases or lipases under oleic acid-induced conditions. The most promising candidates, containing a TG lipase motif (GXSXG) and showing high abundance in the performed screening, were selected for further investigations. Further analysis was therefore restricted to Lpx1p, Ldh1p, Yju3p, Ayr1p, Eht1p, Tsc10p, Ybr056wp, and Ykl050cp (Table 4).

Because previous studies from our laboratory described functions of Tgl3p, Tgl4p, and Tgl5p in addition to their lipase activities, we speculated about dual functions and localizations of other proteins involved in non-polar lipid metabolism (19, 20). Lpx1p is an oleic acid-inducible lipase that localizes to the peroxisomal matrix and is required for normal Px morphology. However, thus far, it has been impossible to identify the reac-
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**TABLE 4**

| ORF     | Gene name | Localization | Motif |
|---------|-----------|--------------|-------|
| YOR084W | LPX1      | Px           | TGL   |
| YBR204C | LDH1      | LD, Mt, Px   | TGL, HYD |
| YKL094W | YLI3      | LD           | TGL (2X) |
| YIL124W | AYR1      | ER, LD, Mt, Cyt | TGL |
| YBR177C | EHT1      | LD, Mt       | TGL   |
| YBR065W | TSC10     | Cyt, ER, Mt  | TGL   |
| YKL050C |           | Unknown      | TGL   |

**TABLE 5**

| Gene | YPO induction factor |
|------|----------------------|
| WT   | TM                   |
| LPX1 | 140                  |
| LDH1 | 4                    |
| YLI3 | 4                    |
| AYR1 | 5                    |
| EHT1 | 3                    |
| TSC10| 2                    |
| YBR056w | 20                 |
| YKL050c | 40               |

**Gene Expression of Candidate Genes in Cells Grown on YPO**—The different protein patterns of LD from cells grown on YPD or YPO, respectively, suggested that different proteins may be active on the different carbon sources. To address this question, the expression levels of the candidate proteins in wild type cultivated on YPD and YPO were compared. All candidate proteins showed an increased expression level on YPO (Table 5). This effect, which we called here the "YPO induction factor," became most obvious for LPX1. This result can be explained by the fact that Lpx1p is a peroxisomal protein. Because Px strongly proliferate in cells grown on YPO, these increased mRNA levels of peroxisomal proteins were not surprising. Interestingly, the unannotated genes YBR056w and YKL050c also showed a very high expression in cells grown on YPO. AYR1 became especially interesting because it showed a 5-fold increase in the relative expression level on YPO. In a second set of experiments, we compared expression levels of candidate genes in the TM cultivated on YPD or YPO, respectively (Table 5). The increase of mRNA levels of LPX1 could again be observed but was less pronounced than in wild type. LDH1, EHT1, YKL050c, and AYR1 also showed significantly elevated levels of mRNA in cells grown on YPO.

**In Vitro Esterase Assays Confirm Hydrolytic Activities of Candidate Proteins**—The eight candidate genes described above were overexpressed in a Δgld3/4/5 background (TM) and purified to perform enzyme analyses. For this purpose, full-length proteins were expressed as fusion proteins with an N-terminal GST tag in *Escherichia coli*. Purified enzymes were analyzed for esterase activities using pNPB as well as pNPA as substrates (Table 6). Lpx1p was successfully purified from peroxisomal fractions, Ayr1p, and Eht1p from LD samples, and Ldh1p and Ybr056wp were isolated from whole cell extracts. Yju3p, Tsc10p, and Ykl050cp could not be purified at sufficient amounts and purity.

Purified enzymes were analyzed for esterase activities using pNPB as well as pNPA as substrates (Table 6). Lpx1p was hydrolyzed pNPB with a $K_m$ of 0.89 mM and a $V_{max}$ of 3.76 μmol/min/mg, which resembled values reported by Thoms et al. (43). Higher activities were found with pNPA as substrate. The highest activities were found for Ayr1p, which cleaved pNPA with a $K_m$ of 3.50 mM and a $V_{max}$ of 18.50 μmol/min/mg, and pNPB, with a $K_m$ of 1.52 mM and a $V_{max}$ of 14.06 μmol/min/mg. All other enzymes tested showed only minor hydrolytic activities with these substrates. $p$-Nitrophenyl palmitate was not converted by any of the enzymes (data not shown). Control assays with the GST tag showed that the tag alone did not affect our results.

**Overexpression of Candidate Genes Affects TG Content of Yeast Cells in the Stationary Phase**—Because TG mobilization is already strongly impaired in a TM strain, additional major lipolytic activities were not expected. To make possible effects of our candidates visible, we chose overexpression of these genes in the TM background rather than additional deletions. Analysis of non-polar lipids from whole cell extracts was performed in cells grown to the stationary phase under these conditions. The TG content in the TM was increased to ~150% of wild type (Fig. 5). Strains overexpressing LPX1*, LDH1*, AYR1*, TSC10*, YBR056w*, and YKL050c* showed a reduction of TG of about 30% compared with the TM. In contrast, overexpression of YLI3* and EHT1* did not significantly change the level of TG (Fig. 5). Lpx1p and Ldh1p had already...
been described as new TG lipases (43–45). As expected, deletion of the respective genes in addition to TGL3, TGL4, and TGL5 did not show an effect on growth in the presence of glucose or oleic acid as a carbon source; nor did it show an effect on the TG content compared with the TM (data not shown).

**In Vivo Mobilization of TG Identifies Ayr1p as Novel TG Lipase**—Changes in the TG content of strains overexpressing putative TG lipases suggested that these proteins also exhibit lipolytic activities in vivo. Therefore, we examined the mobilization of TG in the presence of the fatty acid synthase inhibitor cerulenin with all overexpressing strains described above. Because synthesis of fatty acids is inhibited under these conditions, cells are forced to mobilize TG to ensure sufficient supply for the synthesis of membrane lipids. At the same time, the synthesis of TG is blocked. Degradation of TG was compared both with the wild type and with the TM. Wild type mobilized TG very quickly and showed a nearly complete depletion of TG within 7 h, whereas the TM showed poor mobilization, which leveled off at 80% of the initial value after this time period. This result confirmed previous studies from our laboratory (12). Furthermore, we observed that LPX1 + and AYR1 + showed a significantly altered mobilization rate compared with the TM. The TG level of the AYR1-overexpressing strain dropped to 53%, and the strain overexpressing LPX1 even showed enhanced mobilization to 40% of the starting value (Fig. 6A). All other overexpression strains did not show more mobilization of TG than the TM (Table 7). These results demonstrated that Lpx1p acts as a TG lipase in vivo, confirming previous results obtained in vitro (43). Additionally, the role of Ayr1p as a novel TG lipase of the yeast was fostered. In parallel to the determination of the cellular TG content, growth experiments were performed to exhibit sensitivity to cerulenin. All overexpression strains showed a growth behavior similar to that of TM, which was more sensitive to cerulenin than the wild type (Fig. 6B). In the absence of cerulenin, all strains grew like wild type (data not shown).

In Vitro Enzyme Assays Confirm Ayr1p as Novel TG Lipase—Having shown that Lpx1p and Ayr1p exhibit lipolytic activities in vivo, we also tested them for in vitro TG lipase activity. First, we tested lipase activity of purified Ayr1p and Lpx1p. It had previously been reported that Lpx1p exhibits lipase activity using 1,2-dioleoyl-3-(pyren-1-yl)decanoyl-rac-glycerol as substrate (43). Therefore, Lpx1p served as a positive control. Purified Ayr1p showed an even higher TG lipase activity than Lpx1p (Fig. 7A). To confirm lipase activity of Ayr1p, LD fractions from the AYR1-overexpressing strain were compared with wild type and TM (Fig. 7B). In agreement with our data of the in vivo TG mobilization assays as well as with results obtained with purified enzymes, LD from AYR1 + showed increased TG mobilization activity compared with the TM, reaching the same level as wild type. Michalis-Menten enzyme analysis revealed Ayr1p to

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**TABLE 6**

**In vitro esterase activities**

Esterase activity was measured from purified enzymes using pNPB and pNPA as test substrates. $V_{max}$ and $K_m$ values were calculated using Michaelis-Menten approximations. Data are mean values from three independent experiments with the respective S.D. values.

| Enzyme | Substrate | $V_{max}$ $\mu$mol/min/mg | $K_m$ mmol/min/mg |
|---------|-----------|-----------------------------|------------------|
| Lpx1p   | pNPB      | 3.76 ± 0.54                 | 0.89 ± 0.13      |
| Lpx1p   | pNPA      | 10.94 ± 0.98                | 2.50 ± 0.22      |
| Ldh1p   | pNPB      | 0.90 ± 0.15                 | 0.89 ± 0.15      |
| Ldh1p   | pNPA      | 1.26 ± 0.18                 | 1.78 ± 0.25      |
| Ayr1p   | pNPB      | 14.06 ± 2.23                | 1.52 ± 0.24      |
| Ayr1p   | pNPA      | 18.50 ± 2.76                | 3.50 ± 0.52      |
| Eht1p   | pNPB      | 1.01 ± 0.14                 | 0.88 ± 0.12      |
| Eht1p   | pNPA      | 3.44 ± 0.48                 | 2.06 ± 0.29      |
| Ybr056wp| pNPB      | 1.439 ± 0.22                | 0.81 ± 0.12      |
| Ybr056wp| pNPA      | 7.02 ± 1.26                 | 3.51 ± 0.63      |
| Control: GST tag | pNPB      | 0.35 ± 0.05                 | 0.37 ± 0.05      |

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**FIGURE 5.** Triacylglycerol levels of strains overexpressing candidate lipases. Cells from WT, TM, and strains overexpressing putative novel TG lipases in the TM background were grown in minimal medium until early stationary phase. Total cell and lipid extracts were obtained as described under “Experimental Procedures.” Lipids were separated by TLC and quantified densitometrically. Results shown are the average from at least three independent experiments with the S.D. values (error bars) indicated.
be an active TG lipase hydrolyzing [9,10-^3H]triolein with a $V_{\text{max}}$ of 10.93 pmol/h/mg and a $K_m$ of 61.21 $\mu$M. Linear increase of product formation was observed up to 100 min.

Ayr1p possesses the characteristic GXSSXG motif. To determine the functional significance of this conserved sequence, a point mutation was introduced (S18A), and the purified mutated protein was tested for lipase activity as described above. Replacement of serine by alanine in the lipase motif of mutated protein was tested for lipase activity as described under “Experimental Procedures.”

TABLE 7

| Strain          | TG content after 7 h (% of starting point) |
|-----------------|-------------------------------------------|
| $\Delta$tl3/4/5 (VC) | 80                                        |
| TM + LPX1<sup>+</sup> | 40                                        |
| TM + LDH1<sup>+</sup> | 70                                        |
| TM + YKL050c<sup>+</sup> | 75                                        |
| TM + AYR1<sup>+</sup> | 53                                        |
| TM + EHT1<sup>+</sup> | 70                                        |
| TM + TSC10<sup>+</sup> | 80                                        |
| TM + YBR056c<sup>+</sup> | 70                                        |
| TM + YKL050c<sup>+</sup> | 70                                        |

A major goal of the present study was to identify enzymes that might play a role in the mobilization of TG in the absence of the three major lipases. To address this question, we designed a functional proteome approach, making use of specific fluorescent inhibitors of esterases and lipases as probes. This study led to the detection of a subset of proteins that showed TG hydrolase activity in vitro on isolated LD but also with Px (see Fig. 2). Although these probes specifically recognized catalytic serine residues, detection of false positives could not be excluded. We were also aware that non-catalytic proteins were detected by chance due to the overlap with reactive enzyme bands on the gel. However, the sensitivity and selectivity of the assay was proven to be good enough because all known TG lipases were identified, although Yeh1p, a steryl ester hydrolyase of LD, was not found. Detection of all prominent LD and peroxisomal proteins in the respective samples served as a proof of purity of the isolated organelles. However, our screening detected additional, so far unannotated, proteins on LD or in Px. Our findings complement a previous study from our laboratory aimed at the completion of the LD proteome (3). Data provided in the present study also support the view that many proteins may have multiple localizations and functions in the cell. Regulatory mechanisms governing localization and function of proteins in different compartments have to be taken into account. One recent example for such aspects is the stability, gene expression, and localization of Tgl3p, depending on the availability and the presence of its substrate TG (52).

The list of proteins found in our screening was narrowed to a subset of proteins with putative hydrolase or lipase function (see Table 4) based on computational sequence analysis, assigned functions, and subcellular localizations. Nevertheless, proteins of already annotated localization and function different from non-polar lipid metabolism were included. The reason for this strategy was the discovery of a dual role for the known TG lipases Tgl3p, Tgl4p, and Tgl5p, which also possess high acyltransferase activities (19, 20). Consequently, other proteins might have multiple functions, too, maybe not only on LD but also in other organelles. The short list of putative novel TG

FIGURE 6. In vivo triacylglycerol mobilization in the presence of cerulenin. Cellular TG levels (A) and growth curves (B) of WT (●), TM (●), and the two overexpressing strains TM + AYR1<sup>+</sup> (+) and TM + LPX1<sup>+</sup> (▲) in the presence of cerulenin (inhibitor of fatty acid synthesis) are shown. Data are expressed as relative amounts of TG per culture aliquot with the value at the time point 0 set at 100%. Analyses are representative of at least four independent experiments. Data are mean values with respective S.D. values of about 10%.

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lipases included Lpx1p, Ldh1p, Yju3p, Ayr1p, Eht1p, Tsc10p, Ybr056wp, and Ykl050cp. All of these proteins contain the consensus sequence for a classical lipase (GXSGXG motif) with a classical catalytic triad containing a nucleophile serine. Lpx1p and Ldh1p had been described previously as lipases based on in vitro analysis, and Lpx1p was localized on Px, whereas Ldh1p was identified as an LD component (43–45).

One major question, of course, was the physiological role or relevance of the newly detected hydrolytic/lipolytic enzymes. The TG content analysis of whole cell extracts from overexpression strains indeed proposed a role for Lpx1p, Ldh1p, Ayr1p, Tsc10p, Ybr056wp, and Ykl050cp. These proteins were shown to be expressed at high levels in the absence of the major lipases, at least in the stationary growth phase (see Fig. 5). In vivo mobilization of TG by Lpx1p and Ayr1p confirmed the role of these two proteins as lipases in living cells, whereas all other enzymes did not exhibit lipolytic activities in vivo (see Fig. 6 and Table 7). This result was perfectly in line with in vitro enzyme assays (see Fig. 7). Moreover, these two proteins showed the highest activities when esterases substrates were used (Table 6). Interestingly, a yeast strain lacking not only in the major lipases Tgl3p, Tgl4p, and Tgl5p but also Ayr1p, Lpx1p, or Ldh1p, respectively, still showed mobilization of TG. This finding supported the view that other, more minor lipolytic activities set fatty acids free as supply for energy production and/or synthesis of membrane lipid components. This view was supported by gene expression studies. In these experiments, Lpx1p and Ayr1p were among the most strongly induced genes in the TM grown on oleic acid compared with cells grown on glucose-containing medium (see Table 5). The question remains why TG are mobilized in cells suffering already from an excess of free fatty acids by supply from the medium. Currently, we can only speculate that different pools of lipids exist, which are mobilized upon different requirements. One possibility might be that fatty acids mobilized from TG may serve primarily for phospholipid synthesis or β-oxidation. However, proof for this model is still missing. In a previous study, it had already been reported that growth on oleate led to the accumulation of large amounts of free fatty acids (53). From this finding, it can be assumed that free fatty acids do not play an essential role in regulating the non-polar lipid turnover because TG mobilization occurred normally under these conditions.

In summary, our results suggest that TG turnover is not only catalyzed by the main lipases Tgl3p, Tgl4p, and Tgl5p but is also performed by a subset of lipases and hydrolases with lower activities. Our data demonstrate that the action of such enzymes is strongly dependent on growth conditions and that they may come to the fore when the major enzymes are inactive. Finally, our investigations identified Ayr1p as a novel TG lipase and confirmed the role of Lpx1p as lipase in vivo. These data contribute to more detailed knowledge of non-polar lipid turnover and set the stage to investigate regulatory aspects of non-polar lipid metabolism in more detail.

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