# The *Saccharomyces cerevisiae YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* Genes Encode a Novel Family of Membrane-Anchored Lipases That Are Required for Steryl Ester Hydrolysis

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**Sterol homeostasis in eukaryotic cells relies on the reciprocal interconversion of free sterols and steryl esters. The formation of steryl esters is well characterized, but the mechanisms that control steryl ester mobilization upon cellular demand are less well understood. We have identified a family of three lipases of *Saccharomyces cerevisiae* that are required for efficient steryl ester mobilization. These lipases, encoded by *YLL012/YEH1*, *YLR020/YEH2*, and *TGL1*, are paralogues of the mammalian acid lipase family, which is composed of the lysosomal acid lipase, the gastric lipase, and four novel as yet uncharacterized human open reading frames. Lipase triple-mutant yeast cells are completely blocked in steryl ester hydrolysis but do not affect the mobilization of triacylglycerols, indicating that the three lipases are required for steryl ester mobilization in vivo. Lipase single mutants mobilize steryl esters to various degrees, indicating partial functional redundancy of the three gene products. Lipase double-mutant cells in which the third lipase is expressed from the inducible *GAL1* promoter have greatly reduced steady-state levels of steryl esters, indicating that overexpression of any of the three lipases is sufficient for steryl ester mobilization in vivo. The three yeast enzymes constitute a novel class of membrane-anchored lipases that differ in topology and subcellular localization.**



Sterols are essential lipids of eukaryotic cells. They are present in two major forms: free sterols and steryl esters. Free sterols are synthesized in the endoplasmic reticulum (ER) membrane but are greatly enriched at the plasma membrane, which harbors 90% of the free-sterol pool of a cell (27). Steryl esters, on the other hand, serve as a storage form for fatty acids and sterols that are deposited in intracellular lipid bodies or particles. The conversion of free sterols and acyl coenzymes A to steryl esters is localized to the ER (14). The formation of steryl esters is important to maintain sterol homeostasis, as the steryl ester pool conceptually serves to buffer both excess and a lack of free sterols (12). The genes required for the synthesis of steryl esters in yeast have been identified, and mutants that lack steryl esters are viable, indicating that their synthesis is not essential under standard growth conditions (52, 53). The reverse process, however, how endogenously synthesized steryl esters are mobilized from their stores and hydrolyzed to release free sterols and fatty acids, is less well understood, even though cleavage of this ester bond is generally thought to be catalyzed by a lipase.

Lipases constitute a heterogeneous family of proteins with carboxyl esterase activity and are activated by a lipid-water interface (for reviews, see references 34 and 51). Crystallographic analysis revealed that lipases are remarkably similar in structure despite low overall sequence conservation. They belong to the alpha/beta hydrolase fold family of enzymes with diverse hydrolytic functions. Their catalytic domain consist of a predominantly parallel beta-sheet structure of eight beta sheets connected by helical loops of various lengths that contain the catalytic-triad residues serine, aspartic acid, and histidine (10, 37, 42). The nucleophilic serine of this triad is itself part of the nearly ubiquitous lipase consensus sequence motif GXSXG (17). These three amino acids, assisted by the dipolar oxyanion hole, which stabilizes the charge distribution of the transition state, catalyze the hydrolysis of the ester bond (37).

Only a few gene products with lipolytic activity against neutral lipids have been characterized in *Saccharomyces cerevisiae*. Tgl1 has been proposed to be a triglyceride-specific lipase on the basis of its homology to lipases from humans and rats, but enzymatic activity of Tgl1 against triacylglycerol has never been demonstrated (1). *TGL2*, on the other hand, was found to prevent lethal accumulation of diacylglycerol (DAG) in a DAG kinase mutant *Escherichia coli* strain grown on arbutin, an artificial phosphoglycerol acceptor (49). Tgl2p displays sequence homology to *Pseudomonas* triacylglycerol lipases, and its expression in *E. coli* confers lipolytic activity against triacylglycerol and DAG with short-chain fatty acids (49). The physiological function of *TGL2* in yeast, however, has not been characterized. A third lipase, Tgl3p, is required for mobilization of triacylglycerol in vivo, and the protein confers triacylglycerol lipase activity in vitro (7).

The gene(s) encoding steryl esterase(s) in yeast has not been identified. The steryl ester-hydrolyzing activity is induced under anaerobic conditions and enriched in the membrane pellet of semianaerobically grown yeast cells (46). The activity copurified with a 70-kDa protein, but the enzyme that catalyzes the reaction has not formally been identified (45).

The aim of this study was to identify the presumed steryl ester-hydrolyzing activity in yeast. The yeast genome contains three open reading frames (ORFs) that contain an alpha/beta

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TABLE 1. *S. cerevisiae* strains used in this study

| Strain | Relevant genotype | Source and/or reference |
|---|---|---|
| YRS1533 | BY4742; *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* | EUROSCARF; 50 |
| YRS1972 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*kanMX4* | EUROSCARF; 50 |
| YRS1971 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh2*::*kanMX4* | EUROSCARF; 50 |
| YRS1973 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 tg11*::*kanMX4* | EUROSCARF; 50 |
| YRS1948 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*HIS3MX6 yeh2*::*kanMX4* | This study |
| YRS1837 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*HIS3MX6 tg11*::*kanMX4* | This study |
| YRS1838 | *MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2*::*kanMX4 tg11*::*kanMX4* | This study |
| YRS1840 | *MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1*::*HIS3MX6 yeh2*::*kanMX4 tg11*::*kanMX4* | This study |
| YRS1974 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH1-GFP-HIS3MX6* | This study |
| YRS1861 | *MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ERG6/ERG6-RFP-kanMX6 YEH1/YEH1-GFP-HIS3MX6* | This study |
| YRS2086 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH2-GFP-HIS3MX6* | This study |
| YRS1858 | *MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 TGL1-GFP-HIS3MX6* | This study |
| YRS2105 | *MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ERG6/ERG6-RFP-kanMX6 TGL1/TGL1-GFP-HIS3MX6* | This study |
| YRS2085 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-YEH1* | This study |
| YRS2083 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-YEH2* | This study |
| YRS2084 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-TGL1* | This study |
| YRS1953 | *MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS trp1Δ* | This study |
| YRS1956 | *MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4tgl1*::*kanMX4* | This study |
| YRS2090 | *MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh2*::*kanMX4 tgl1*::*kanMX4 YEH1-GFP-TRP1* | This study |
| YRS2091 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 tgl1*::*kanMX4 YEH2-GFP-TRP1* | This study |
| YRS2092 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4 TGL1-GFP-TRP1* | This study |
| YRS2161 | *MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh2*::*kanMX4 tgl1*::*kanMX4 TRP1-PGAL1-GFP-YEH1* | This study |
| YRS2162 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 tgl1*::*kanMX4 TRP1-PGAL1-GFP-YEH2* | This study |
| YRS2163 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4 TRP1-PGAL1-GFP-TGL1* | This study |



hydrolase-associated lipase region, as classified by the pfam database of protein families. To determine whether one or more of these genes is required for steryl ester hydrolysis, we used an in vivo assay to monitor the mobilization of radiolabeled steryl ester in mutant strains lacking one, two, or all three of these candidate lipases. The results of this analysis indicate that together these three lipases account for all of the steryl ester-hydrolyzing activity that is present in yeast. Unexpectedly, all three yeast lipases appear to be membrane anchored, which makes them the first, to our knowledge, membrane-anchored neutral lipid lipases to be described. Their subcellular distribution and possible membrane topology with respect to the localization of the active site are discussed.

## MATERIALS AND METHODS

**Yeast strains and growth conditions.** The yeast strains used in this study are listed in Table 1. Strains bearing single deletions of nonessential genes were obtained from EUROSCARF (www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html) (50). Strains were cultivated in YPD rich medium (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose) or in minimal medium. Selection for the *kanMX4* marker was on medium containing G418 (Gibco BRL, Life Technologies) at 200 μg/ml. Double- and triple-mutant strains were generated by crossing of single mutants and by gene disruption with the PCR deletion cassettes (31) and the primers listed in Table 2. Wild-type and double- and triple-mutant strains were converted to tryptophan auxotrophy with plasmid pNKY1009 (*trp1::URA3*) and converted back to uracil auxotrophy by selection on FOA medium (2). Yeast was transformed by lithium acetate (25).

**Green fluorescent protein (GFP) tagging, subcellular fractionation, and Western blot analysis.** N- and C-terminal GFP tagging of *YEH1*, *YEH2*, and *TGL1* was performed by homologous recombination with the PCR fusion cassette from pFA6a-HIS3MX6-PGAL1-GFP(S65T), pFA6a-TRP1-PGAL1-GFP(S65T), pFA6a-GFP(S65T)-TRP1, and pFA6a-GFP(S65T)-HIS3MX6 (31) and the primers listed in Table 2. The resulting PCR fragments were transformed into wild-type strain BY4742 or BY4741, and correct integration of the fusion cassette was confirmed by colony PCR with the control primers listed in Table 2.

Protein concentrations were determined by the method of Lowry et al. (33), with the Folin reagent and bovine serum albumin as the standard. Proteins were precipitated with 10% trichloroacetic acid (TCA), resuspended in sample buffer, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Western blots were probed with rabbit antisera against GFP (1:5,000; Torrey Pines Biolabs, Inc., Houston, Tex.), Kar2 (1:5,000; M. Rose, Princeton University), Erg6 and porin (both 1:10,000; kind gifts from G. Daum, Graz University

TABLE 2. Primers used in this study

| Primer name | Sequence (5′–3′)[a] | Purpose |
|---|---|---|
| YEH1; fwdYLLko | TTTTAATATAGATGGCTGCAGATAAAGTAATAGTTTTATAcggatccccgggttaattaa | Forward primer for *YEH1*::*HIS3MX6* cassette |
| YEH1; revYLLko | AAATGTATCGTTATGCACGGTGATGTCCCAACTCCCGACAgaattcgagctcgtttaaac | Reverse primer for *YEH1*::*HIS3MX6* cassette |
| YEH1; upYLLreg | ACGTATCGCAGGCAATGTGC | Forward control primer for gene deletion |
| YEH1; F2yllGFP | ACAGAGGTGGAAACGGAGCTGGAAATGGTTGCTGAGAAGcggatccccgggttaattaa | Forward primer for C-terminal GFP tagging of *YEH1* |
| YEH1; R1yllGFP | AAATGTATCGTTATGCACGGTGATGTCCCAACTCCCGACAgaattcgagctcgtttaaac | Reverse primer for C-terminal GFP tagging of *YEH1* |
| YEH1; regYLLfwd | ACGCATCAAAGGCCCCAAAT | Forward control primer for C-terminally tagged *YEH1* |
| YEH1; F4Yehprom | AATACTTATTTTTTAATATAGATGGCTGCAGATAAAGTAAgaattcgagctcgtttaaac | Forward primer for N-terminal PGAL1-GFP tag |
| YEH1; R5yehgfpN | TAAATTCCTAGCTCTTTTCAACACCGCAGAAACACCTGCtttgtatagttcatccatgc | Reverse primer for N-terminal PGAL1-GFP tag |
| YEH1; revYEHreg | TTGTCATGTTCCAGATCGAGG | Reverse control primer for N-terminal PGAL1-GFP tag |
| YEH2; F2ylrGFP | ATAGATAGAATTGGTAAGCCAATGATAGAAAATTTGAGGcggatccccgggttaattaa | Forward primer for C-terminal GFP tagging of *YEH2* |
| YEH2; R1ylrGFP | TTTACGTGTAACATCTACAGATACATATATCCGTATATACcaattcgagctcgtttaaac | Reverse primer for C-terminal GFP tagging of *YEH2* |
| YEH2; regYLRmid | GTGAGGCTAATGGCAAAAGG | Control primer for C-terminal tag |
| YEH2; F4YLRprom | TTTCTCATCAAAGGTATAATTAAAATTGTTGGGAAATACTgaattcgagctcgtttaaac | Forward primer for N-terminal PGAL1-GFP tag |
| YEH2; R5YLRgfpN | CACCAACCGCTGAACCTCATCAACCACCTTATTTACTGCtttgtatagttcatccatgc | Reverse primer for N-terminal PGAL1-GFP tag |
| YEH2; revYLRreg | GGGCGTTGTGGAAGATATTGA | Reverse control primer for N-terminal PGAL1-GFP tag |
| YEH2; regYLR450up | CCACCCGTGTTTCCTAGGTT | Forward control primer for gene deletion |
| TGL1; F2tglGFP | CGACAACTAGATGCCAACTCTTCGACAACTGCGCTGGATcggatccccgggttaattaa | Forward primer for C-terminal GFP tagging of *TGL1* |
| TGL1; R1tglGFP | GATATTAAGACTTCTTATGAAATCCATTTATTGTGTATAgaattcgagctcgtttaaac | Reverse primer for C-terminal GFP tagging of *TGL1* |
| TGL1; regTGL250up | TCCCGGATACTTATTGAAGCTA | Forward control primer for C-terminal GFP tag and for gene deletion |
| TGL1; F4TGLprom | AACAAGGAAAGAAGAAAGAAAACAATTCGAACAAAACTTTgaattcgagctcgtttaaac | Forward primer for N-terminal PGAL1-GFP tag |
| TGL1; R5TGLgfpN | ATCTGTCGATAATCTGCCTAAAAAGGGGAAGTATGCtttgtatagttcatccatgc | Reverse primer for N-terminal PGAL1-GFP tag |
| TGL1; revTGLreg | TACCAAATGATCCTCGACAG | Reverse control primer for N-terminal PGAL1-GFP tag |
| HIS5; His5contrr | TACGGGCGACAGTCACATC | Reverse control primer for *HIS3MX6* |
| GFP; revGFPkontr | TTCGGGCATGGCACTCTTGA | Reverse control primer for GFP |

[a] Sequences in uppercase indicate homology to the genome of *S. cerevisiae*, and those in lowercase indicate homology to the PCR cassette used.

of Technology), Sed5p (1:3,000; a kind gift from H. Pelham, MRC Cambridge), Tlg1p (1:1,000; a kind gift from H. Pelham), Mnn1p (1:400, a kind gift from T. Graham, Vanderbilt University, Nashville, Tenn.), Pma1 (1:50,000), Gas1 (1:5,000, a kind gift from A. Conzelmann, University of Fribourg), or mouse anti-Pho8p (2 μg/ml; Molecular Probes).

Fractionation on an Accudenz density gradient was performed essentially as described by Cowles et al. (16). One hundred optical density (OD) units of exponentially growing cells were collected by centrifugation (3,000 × *g*, 10 min), resuspended in 100 mM Tris (pH 9.4)–10 mM dithiothreitol, and incubated for 10 min at 30°C. Cells were pelleted (3,000 × *g*, 5 min) and resuspended in 0.2× YPD medium–0.6 M sorbitol–10 mM $KP_i$, pH 7.5. Cells were converted to spheroplasts by incubation with 16 U of Zymolyase (Seikagaku Corporation, Tokyo, Japan) for 20 min at 30°C. Spheroplasts were pelleted (4,000 × *g*, 20 min), resuspended in lysis buffer A (0.2 M sorbitol–50 mM potassium acetate–20

mM HEPES [pH 6.8]–2 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride [PMSF] supplemented with a protease inhibitor cocktail [Complete; Roche-Diagnostics, Rotkreuz, Switzerland]), and lysed with a Dounce homogenizer. The homogenate was layered on top of an 8 to 43% Accudenz gradient (Accurate Chemical and Scientific Corp.) prepared in nine steps in lysis buffer A as described by Cowles et al. (16). The gradient was then centrifuged in a swing-out rotor (Sorvall TH641; Kendro Laboratory Products, Asheville, N.C.) at 170,000 $\times$ *g* for 18 h at 4°C. Eleven fractions of equal volume were collected from the top of the gradient, proteins were precipitated, and the distribution of marker proteins was analyzed by Western blotting.

To determine the membrane association of the GFP-tagged lipases, exponentially growing cells were pelleted by centrifugation (3,000 $\times$ *g*, 10 min), resuspended in lysis buffer A, converted to spheroplasts, and lysed as described above. The homogenate was precleared by centrifugation at 500 $\times$ *g* for 10 min and then separated into pellet (P13) and supernatant (S13) fractions by centrifugation at 13,000 $\times$ *g* for 20 min. The S13 fraction was then further separated into a 30,000 $\times$ *g* pellet (P30) and supernatant (S30) by centrifugation at 30,000 $\times$ *g* for 30 min. The S30 was fractionated again by centrifugation at 100,000 $\times$ *g* for 30 min to obtain a 100,000 $\times$ *g* pellet (P100) and supernatant (cytosol). All centrifugation steps were performed at 4°C.

To determine the membrane association and topology of the GFP-tagged lipases, detergent and salt extractions and proteinase K protection experiments were performed essentially as previously described (47). Detergent and salt extractions of microsomal membranes (P13) were performed by incubating 50 μg of the microsomal fraction with either 1% Triton X-100, 7% SDS, 1 M NaCl, or 0.1 M $Na_2CO_3$ in lysis buffer A without protease inhibitors for 30 min on ice. Samples were then centrifuged at 13,000 $\times$ *g* for 30 min, and proteins from the pellet and supernatant fractions were analyzed by Western blotting. For the proteinase K protection experiment, microsomes (50 μg) were incubated with 9, 14, or 28 μg of proteinase K per ml for 30 min on ice. The reaction was stopped by addition of PMSF (5 mM), and proteins were precipitated with 10% TCA. The pellet was dissolved in sample buffer, heated to 95°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Vacuoles and lipid particles were isolated by flotation as previously described (29). Briefly, spheroplasts were resuspended in lysis buffer B (12% Ficoll, 10 mM MES-Tris [pH 6.9], 0.2 mM EDTA) and lysed with a Dounce homogenizer. The lysate was cleared by centrifugation at 5,000 $\times$ *g* for 5 min. The supernatant was placed at the bottom of an ultracentrifuge tube and overlaid with lysis buffer. Lipid particles were subjected to flotation by centrifugation at 100,000 $\times$ *g* for 1 h. The floating fraction was removed, diluted with lysis buffer, and placed at the bottom of a second ultracentrifuge tube. The sample was overlaid with 8% Ficoll–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA and centrifuged again at 100,000 $\times$ *g* for 1 h. The second flotation product was removed and diluted with 0.6 M sorbitol–8% Ficoll–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA. The sample was then placed at the bottom of a third ultracentrifuge tube filled with 0.25 M sorbitol–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA and again subjected to flotation by centrifugation at 100,000 $\times$ *g* for 1 h. The third flotation product was greatly enriched in lipid particle markers, whereas the pellet was enriched in vacuolar marker proteins.

Plasma membrane was enriched by centrifugation through a sucrose step gradient as previously described (43). Cells were harvested, resuspended in lysis buffer D (20 mM Tris [pH 8.5], 5 mM EDTA, 1 mM PMSF), and broken by vigorous agitation with 0.3-mm-diameter glass beads with a Merkenschlager homogenizer (B. Braun Biotech, Melsungen, Germany) equipped with $CO_2$ cooling. The lysate was cleared by centrifugation (5,000 $\times$ *g*, 10 min), and membranes were pelleted by centrifugation (20,000 $\times$ *g* 10 min). Membranes were resuspended in TEDG (10 mM Tris HCl [pH 7.5], 1 mM EDTA, 20% glycerol, 0.2 mM dithiothreitol) and layered on top of a sucrose step gradient (53 to 43%, prepared in TEDG). This gradient was centrifuged at 120,000 $\times$ *g* for 2 h, and the membranes from the interface were collected with a syringe. The plasma membrane fraction was diluted in 10 mM Tris–1 mM EDTA and finally pelleted by centrifugation (20,000 $\times$ *g*, 20 min).

**Fluorescence microscopy.** In vivo localization of GFP-tagged versions of Yeh1, Yeh2, and Tgl1 was performed by fluorescence microscopy with a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam charge-coupled device camera and AxioVision 3.1 software. Lipid droplets were visualized with red fluorescent protein (RFP)-tagged Erg6 as a marker protein (24), and nuclei were visualized by 4′,6′-diamidino-2-phenylindole (DAPI) staining.

**In vivo neutral lipid mobilization.** The neutral lipid pool was labeled by incubating the cells with 10 μCi of [$^3$H]palmitic acid (American Radiolabeled Chemicals Inc., St. Louis, Mo.) per ml for 16 h at 24°C. Cells were then washed and diluted in liquid YPD medium containing either 10 μg of cerulenin (ICN Biomedicals, Irvine, Calif.) per ml or 30 μg of terbinafine (a kind gift from N. Ryder [Novartis Research Institute, Vienna, Austria]) per ml. Aliquots of cells were removed at the time points indicated. Cells were frozen and broken with glass beads, and lipids were extracted with chloroform-methanol (1:1, vol/vol). Radioactivity in the lipid extract was determined by scintillation counting, and equal counts were brought to dryness. Lipids were separated on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) developed in petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol) and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. TLC plates were then exposed to a tritium-sensitive screen and visualized with a phosphorimager (Bio-Rad Laboratories, Hercules, Calif.).

**Overexpression and quantification of the endogenous steryl ester pool.** The strains indicated were cultivated in rich medium containing either glucose (repressing conditions) or galactose (inducing conditions) as a carbon source. The neutral lipid pool was labeled to steady-state levels by incubating the cells with 10 μCi of [$^3$H]palmitic acid (American Radiolabeled Chemicals Inc.) per ml for 16 h at 24°C. Cells were diluted in YPD or YPGal (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological], 2% galactose) liquid medium containing terbinafine (30 μg/ml). Aliquots of cells were removed at the time points indicated, and 3 OD units were used for Western blot analysis. The remaining cells (~20 OD units) were washed and broken, and lipids were extracted as described above. Equal counts were brought to dryness, and lipids were separated on TLC plates, developed in petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol), and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer.

**In vitro assay for steryl ester hydrolase activity.** Overnight cultures of strains were broken with glass beads, and the membrane pellet was solubilized with 1% Triton X-100 in 50 mM Tris (pH 7.0)–10% glycerol–1 mM $MnCl_2$ for 60 min at 4°C. Steryl ester hydrolase activity was determined with 100 to 300 μg of solubilized protein and cholesterol–[1-$^{14}$C]oleate (specific activity, 50 to 60 mCi/mmol; American Radiolabeled Chemicals Inc.) as the substrate as previously described (46). The assay was performed at 30°C for 60 min in a reaction mixture containing 100 mM phosphate buffer (pH 6.9)–0.3% Triton X-100–26 nmol substrate. The reaction was stopped by addition of chloroform-methanol (1:2; vol/vol), lipids were extracted and separated by TLC with petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol) as a solvent system, and liberated fatty acids were quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. TLC plates were then visualized with a phosphorimager (Bio-Rad Laboratories).

## RESULTS

***YLL012*/*YEH1*, *YLR020*/*YEH2*, and *TGL1* are required for efficient in vivo mobilization of steryl esters.** To identify the gene(s) required for steryl ester hydrolysis in yeast, we undertook a candidate gene approach and examined the rate of in vivo mobilization of steryl esters in mutants lacking putative lipase-encoding genes. Strains harboring deletions of the genes of interest were labeled for 16 h in the presence of [$^3$H]palmitate and mobilization of storage lipids was monitored after diluting cells in medium containing terbinafine, an inhibitor of sterol biosynthesis (26). Samples were withdrawn after different time points, lipids were extracted, and the relative content of steryl esters and triacylglycerols was analyzed after TLC separation of neutral lipids. This analysis revealed a reduced rate of steryl ester mobilization in cells lacking *YLL012*, hereafter referred to as *YEH1* (yeast steryl ester hydrolase 1). Yeh1 belongs to a family of three yeast ORFs that contain an alpha/beta hydrolase-associated lipase region as classified by the pfam database of protein families (release 12.0; http://pfam.wustl.edu). Lack of the other two candidate ORFs bearing this hydrolase-associated lipase region, *YLR020*/*YEH2* and *TGL1*, on the other hand, did not significantly affect the rate of steryl ester mobilization under these assay conditions (Fig. 1).

Steryl ester hydrolysis is reduced in *yeh1*Δ mutant cells but not completely abolished, indicating the presence of additional, redundant, activities. To test whether *YEH2* and/or *TGL1* contribute to the residual steryl ester-hydrolyzing activ-

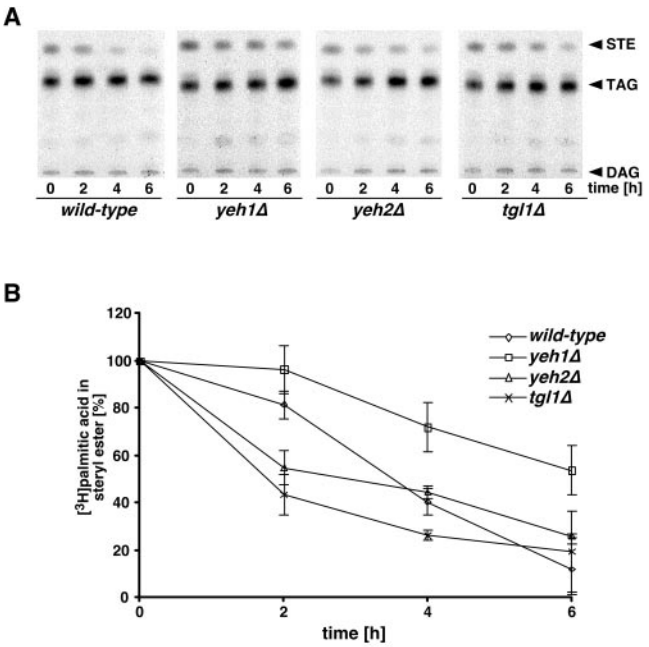



FIG. 1. Lack of *YEH1* affects the efficiency of steryl ester mobilization in vivo. (A) Wild-type (BY4742, YRS1533) and *yeh1*Δ (YRS1972), *yeh2*Δ (YRS1971), and *tgl1*Δ (YRS1973) mutant cells were labeled for 16 h with [$^3$H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0, 2, 4, and 6 h after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. TAG, triacylglycerol; STE, steryl esters. (B) The content of [$^3$H]palmitic acid-labeled steryl ester was quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations of two independent experiments.

ity observed in *yeh1*Δ mutant cells, double- and triple-mutant strains were generated and tested for steryl ester hydrolysis in vivo. This analysis revealed that steryl ester hydrolysis is substantially reduced in any one of the three double mutants and completely blocked in a *yeh1*Δ *yeh2*Δ *tgl1*Δ triple mutant (Fig. 2). Interestingly, steryl ester levels steadily increased in the triple mutant, indicating that steryl ester synthesis is ongoing while their mobilization is completely blocked. These results indicate that all of the members of this lipase family contribute to steryl ester hydrolysis in vivo and that the lack of all three lipases cannot be compensated for by other activities. The observation that the triple-mutant strain grows like wild-type cells furthermore indicates that steryl ester hydrolysis is a nonessential process under standard growth conditions (data not shown).

**Lack of *YEH1*, *YEH2*, and *TGL1* does not affect triacylglycerol mobilization.** To examine the apparent substrate specificity of the three lipases in vivo, single-, double-, and triple-mutant cells were labeled with [$^3$H]palmitate, but this time neutral lipid mobilization was induced by inhibiting de novo fatty acid synthesis with cerulenin (38). Under these assay conditions, wild-type cells efficiently mobilized triacylglycerols over a 3-h time period (Fig. 3). The lipase mutants, on the other hand, did not affect triacylglycerol mobilization, indicating that this family of lipases is important for efficient in vivo mobilization of steryl esters rather than triacylglycerols.

***YEH1*, *YEH2*, and *TGL1* are paralogues of the human acid lipase family.** Sequence analysis of Yeh1, Yeh2, and Tgl1 revealed that they are paralogues of the human acid lipase family, which contains the lysosomal acid lipase A, LIPA/LAL, and the secreted gastric lipase LIPF (5; for a review, see reference 34). Further inspection of the human genome sequence revealed the presence of four additional members of this lipase family, LIPL1 to LIPL4, that together with LIPF and LIPA form a lipase gene cluster on chromosome 10q23 (http://www.ensembl.org/). Multiple-sequence alignment of the three yeast steryl ester hydrolases with the two human acid lipases, LIPA and LIPF, and two of the predicted lipases, LIPL1 and LIPL3, reveals conservation of the catalytic-triad residues (Fig. 4). LIPL2 and LIPL4 were not included in this alignment as their coding sequences have not been completely predicted.

The alignment further reveals complete conservation of the GXSXG lipase motif in five of the seven lipases. In Yeh1, the first glycine of this lipase motif is replaced by a serine and in Yeh2 it is replaced by an alanine. The functionally important cysteine residues of LIPA (Cys248 and Cys257), however, are conserved in Yeh1 only (30, 40). The predicted molecular masses of these lipases range from 62 to 66 kDa, which are in good agreement with the experimentally determined 70-kDa molecular mass of the enriched yeast steryl ester hydrolase activity (45). Interestingly, all three yeast proteins are predicted to be integral membrane proteins with one to three

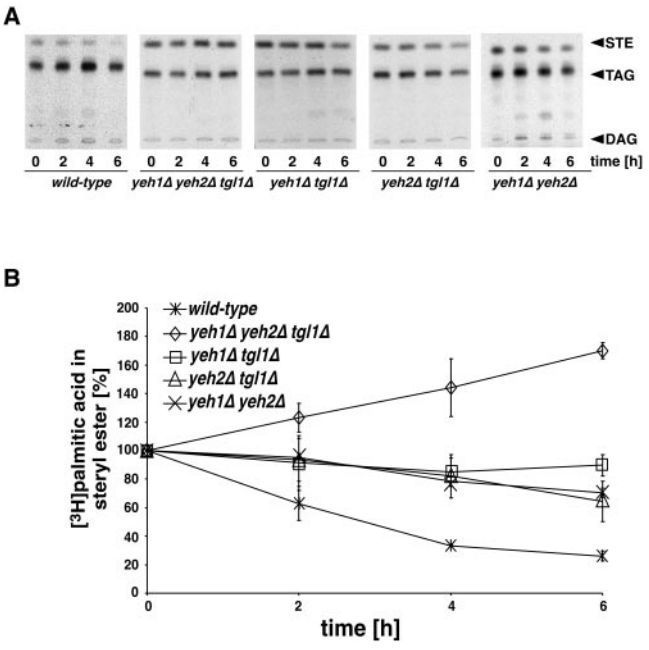



FIG. 2. *yeh1*Δ *yeh2*Δ *tgl1*Δ triple-mutant cells fail to mobilize steryl esters in vivo. (A) Lipase triple-mutant (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840) and double-mutant (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948) cells were labeled for 16 h with [$^3$H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. TAG, triacylglycerol; STE, steryl esters. (B) The content of [$^3$H]palmitic acid-labeled steryl ester was quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments.

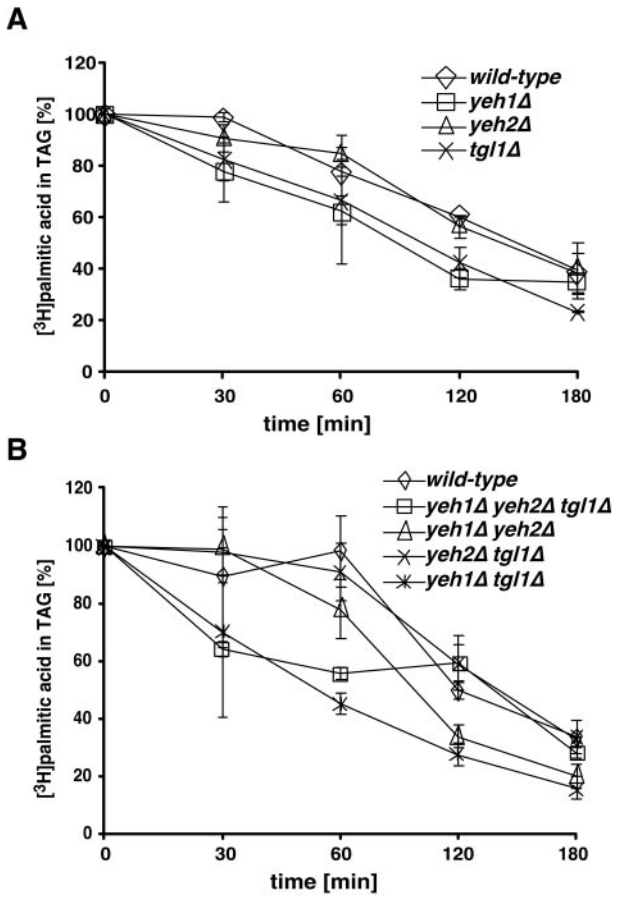



FIG. 3. *YEH1*, *YEH2*, and *TGL1* are not required for efficient mobilization of triacylglycerol. (A) Wild-type (BY4742, YRS1533) and *yeh1*Δ (YRS1972), *yeh2*Δ (YRS1971), and *tgl1*Δ (YRS1973) mutant cells were labeled for 16 h with [$^3$H]palmitic acid, and the kinetics of triacylglycerol (TAG) mobilization was analyzed after dilution of the cells in medium containing cerulenin. Lipids were extracted and analyzed by TLC, and the relative content of [$^3$H]palmitic acid-labeled TAG was quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments. (B) Wild-type (BY4742, YRS1533) and lipase triple-mutant (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840), and double-mutant (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948) cells were labeled for 16 h with [$^3$H]palmitic acid, and the kinetics of TAG mobilization was analyzed as described for panel A.

putative transmembrane domains and one to three potential N-glycosylation sites. The number and position of the predicted membrane domains, however, varied considerably, depending on the type of algorithm used (Table 3). The fact that the results of these predictors vary considerably may be explained in part by the fact that these proteins accommodate hydrophobic substrates into a cleft of the protein that may be mistaken as a potential transmembrane domain.

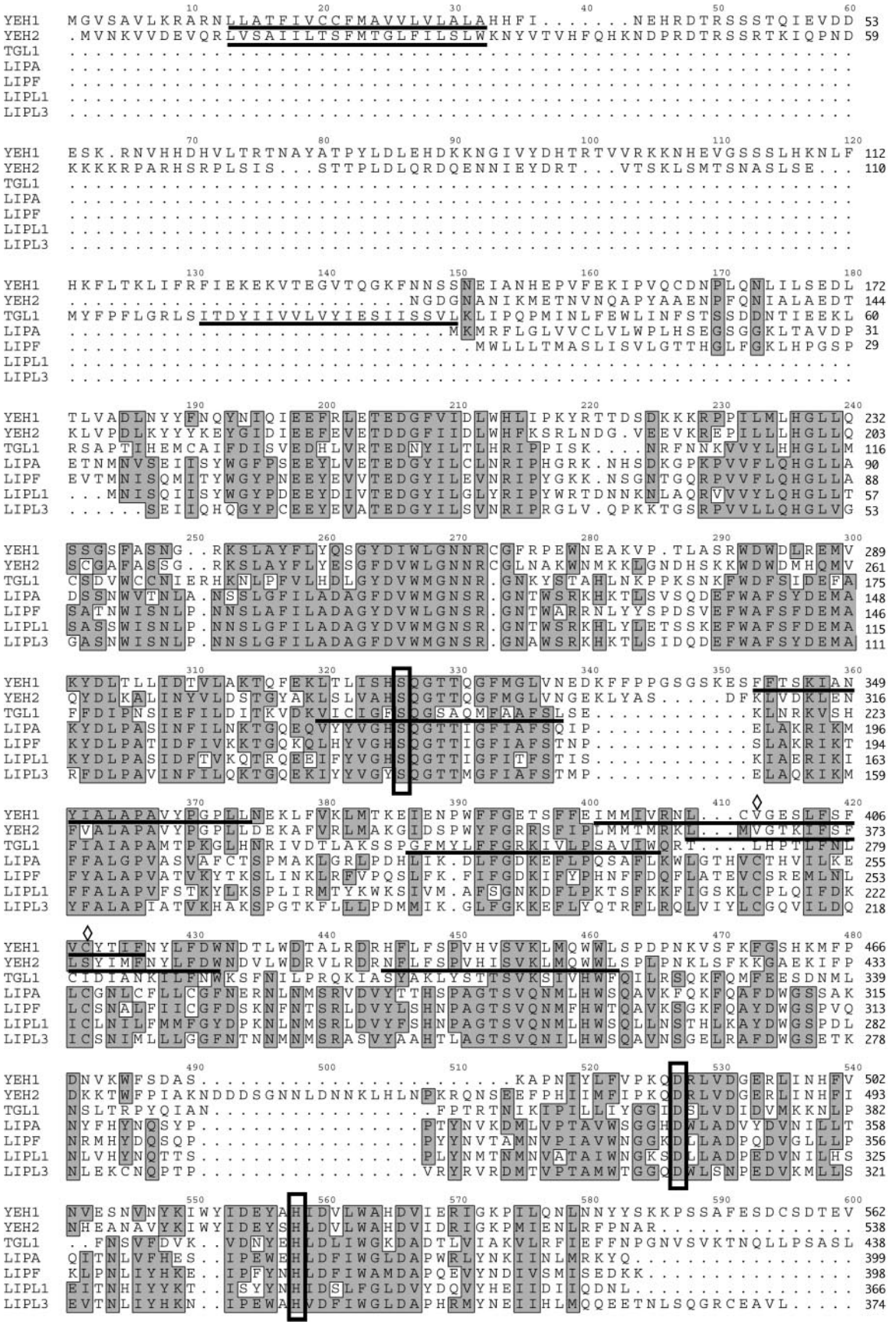
**Subcellular localization of the three yeast lipases.** To determine the subcellular location of the three lipases, their genomic copies were tagged to produce fusion proteins with C-terminally appended GFP by homologous recombination. All fusions were functional, as revealed by their in vivo activity in a double-mutant background (Fig. 5A). Fluorescence microscopy of strains expressing Yeh1-GFP and Tgl1-GFP revealed staining of punctate intracellular structures that colocalized with RFP-tagged Erg6, a marker protein for lipid particles (8, 24) (Fig. 5B). The C-terminally tagged copy of Yeh2 failed to give a fluorescence signal even though the fusion protein was expressed abundantly, as determined by Western blotting with anti-GFP antibody (Fig. 5C). Lack of fluorescence of the Yeh2-GFP fusion might indicate that the GFP domain of the fusion protein may not fold correctly to form the chromophore or that the half-life of the protein may be too short for its formation (22). The subcellular localization of Yeh2 was thus determined by using an N-terminally GFP-tagged version of Yeh2, driven by a galactose-inducible *GAL1* promoter. To localize GFP-Yeh2, cells were first grown in raffinose-containing medium and then shifted to galactose medium for 4 h to induce expression of the reporter. Examination of these cells revealed punctate staining of the cell periphery, suggestive of a possible plasma membrane localization of GFP-Yeh2 (Fig. 5B).

To determine whether these three lipases are indeed membrane associated, as predicted on the basis of their sequences, their fractionation properties were examined by differential centrifugation. All three proteins were slightly enriched (about threefold) in the 13,000 $\times$ *g* membrane pellet but were not detectable in the cytosol, consistent with the membrane association of all three lipases (Fig. 5C).

To confirm the localization of Yeh1-GFP to lipid particles, lipid particles and associated vacuoles were isolated by subcellular fractionation and the degree of enrichment of Yeh1-GFP in these fractions was determined by Western blot analysis. Consistent with its microscopic localization, Yeh1-GFP is ~11-fold enriched in the lipid particle fraction but hardly detectable in the vacuolar fraction (Fig. 5D). The second anti-GFP-reactive band, which migrates faster than the major ~93-kDa band, is likely due to N-terminal degradation of Yeh1-GFP. This lipid particle fraction is highly enriched in its marker protein, Erg6 (~350-fold). We did not attempt to localize Tgl1 by fractionation because it is known to be a major lipid particle protein (8).

Since C-terminally GFP-tagged Yeh2 could not be localized by microscopy, its fractionation properties on an Accudenz density gradient were examined. Western blot analysis of Yeh2-GFP yields two major bands of ~91 and ~81 kDa, which appear to be due to some as yet unidentified modification of the protein. These two major and probably mature forms of Yeh2-GFP (Fig. 5C) were enriched in fractions 2 and 3 of the Ac-

FIG. 4. Sequence alignment of the three yeast lipases with the mammalian acid lipase family. Sequence alignment of yeast lipases Yeh1 (Q07804), Yeh2 (Q07950), and Tgl1 (P34163) with mammalian acid lipase family members LIPA (P38571), LIPF (P07098), LIPL1 (Q5W064), and LIPL3 (Q5VYY2). Residues of the catalytic triad are boxed. The two functionally important cysteine residues of LIPA are indicated by open diamonds. The positions of potential transmembrane domains in the three yeast lipases, as predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and Phobius (http://phobius.cgb.ki.se/), are indicated by horizontal lines. Alignment was performed with ClustalW (http://www.ebi.ac.uk/clustalw/), and regions of homology were drawn by JavaShade (http://industry.ebi.ac.uk/JavaShade/).



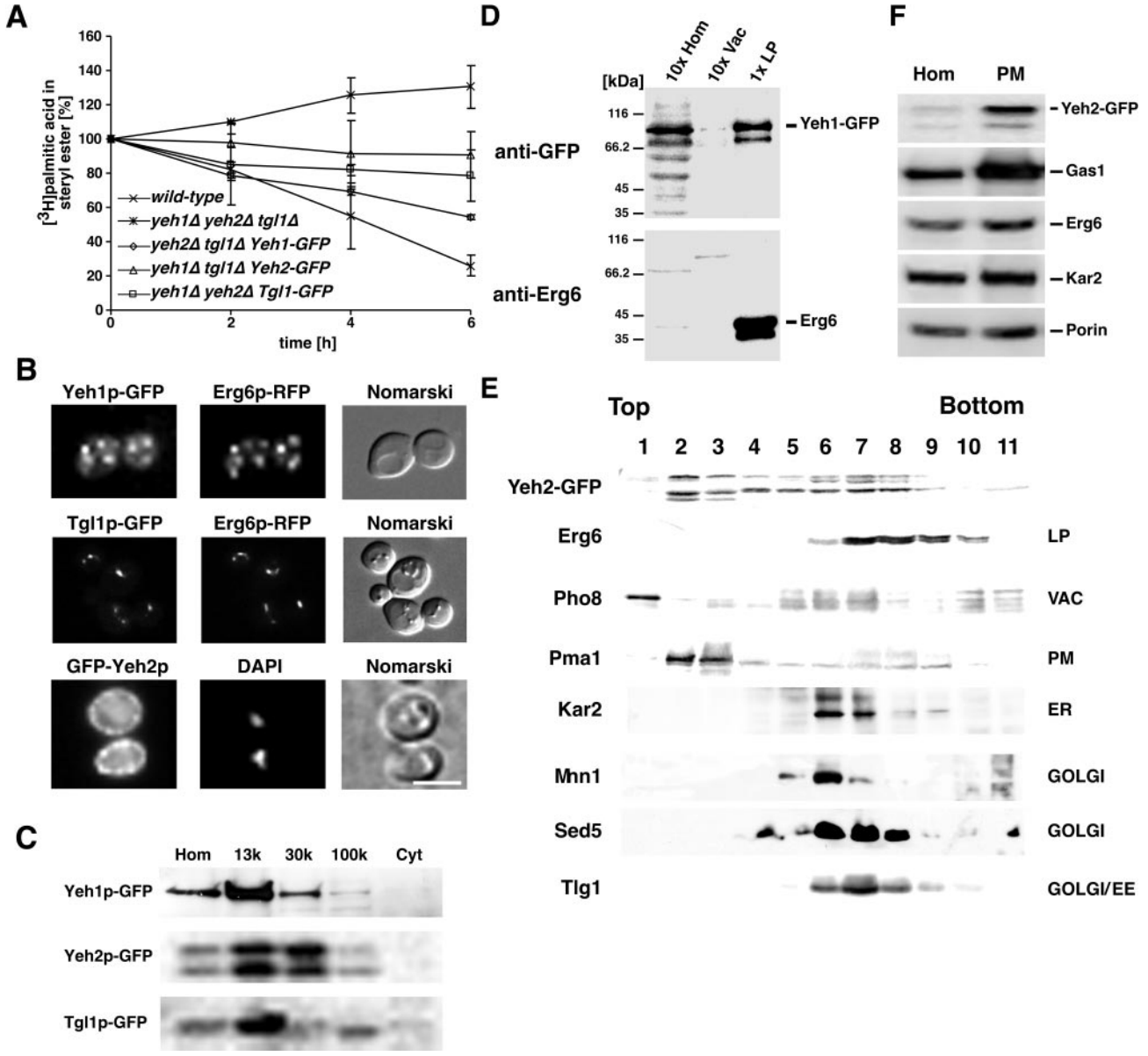



FIG. 5. Subcellular localization of Yeh1, Yeh2, and Tgl1. (A) Functionality of the GFP-tagged lipases. Wild-type (YRS1953), triple-mutant (YRS1956), and double-mutant cells in which the remaining lipase is C-terminally tagged with GFP (YRS2090, YRS2091, and YRS2092) were labeled for 16 h with [$^3$H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0, 2, 4, and 6 h after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. The content of [$^3$H]palmitic acid-labeled steryl ester was quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations of two independent experiments. (B) In vivo localization of Yeh1-GFP, GFP-Yeh2, and Tgl1-GFP. Heterozygous diploid cells expressing chromosomally tagged versions of Yeh1-GFP and Erg6-RFP (YRS1861) or Tgl1-GFP and Erg6-RFP (YRS2105) were grown in YPD medium and examined by fluorescence microscopy. N-terminally GFP-tagged Yeh2 (YRS2085) was localized after induction in galactose-containing medium for 4 h and stained for DNA with DAPI. Bar, 5 μm. (C) Yeh1, Yeh2, and Tgl1 are membrane associated. Homogenates (Hom) from cells expressing the GFP-tagged lipases were fractionated by differential centrifugation to yield 13,000 × *g* (13k), 30,000 × *g* (30k), and 100,000 × *g* (100k) membrane pellets and cytosolic supernatants (Cyt). Ten-microgram samples of proteins from each fraction were separated by electrophoresis, blotted, and probed with an anti-GFP antibody. (D) Yeh1 is enriched in lipid particles. Haploid cells expressing Yeh1-GFP (YRS1974) were cultivated in rich medium. Vacuoles and lipid particles were isolated by flotation. Homogenate (Hom, 10 μg), vacuole (Vac, 10 μg), and lipid particle (LP, 1 μg) proteins were separated by electrophoresis, blotted, and probed with antibodies against GFP (anti-GFP) and Erg6 (anti-Erg6). The positions of molecular size markers are indicated to the left. (E) Yeh2-GFP cofractionates with plasma membrane markers. Membranes from cells expressing Yeh2-GFP (YRS2086) were fractionated on an Accudenz density gradient, and the presence of Yeh2-GFP was detected by Western blotting with an anti-GFP antibody. The distribution of marker proteins in individual fractions from the gradient was determined by Western blotting with antibodies to Erg6, a marker protein for lipid particles (LP) (8); Pho8–anti-Pho8p, a vacuolar phosphatase (VAC); Pma1, the plasma membrane proton pump (PM); Kar2, an ER luminal chaperone (ER) (15); Mnn1, a medial-Golgi α1,3-mannosyltransferase (GOLGI) (20); Sed5, a t-SNARE of the *cis*-Golgi (21); and Tlg1, an endosomal-Golgi t-SNARE (GOLGI/EE) (23). (F) Yeh2-GFP is enriched in the plasma membrane fraction. Plasma membrane was enriched by centrifugation of membranes from cells expressing Yeh2-GFP (YRS2086) on a sucrose step gradient and probed for the presence of the GFP epitope, the GPI-anchored plasma membrane protein Gas1, the lipid particle marker Erg6, the ER luminal protein Kar2, and porin, as a marker protein for mitochondria.

cudenz gradient. The same fractions were also enriched for the plasma membrane proton-pumping ATPase Pma1 (Fig. 5E). Thus, fractionation of Yeh2-GFP expressed from its endogenous promoter is consistent with plasma membrane localization.

To further confirm the localization of Yeh2, plasma membrane from a strain expressing Yeh2-GFP was enriched by fractionation and the relative enrichment of Yeh2-GFP was determined by Western blot analysis. Similar to the glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein Gas1, Yeh2 is enriched about sixfold in the plasma membrane fraction, consistent with plasma membrane localization of Yeh2. Other marker proteins, such as the mitochondrial porin or ER luminal Kar2 or Erg6, however, were not enriched in this fraction (Fig. 5F). These data indicate that all three lipases are membrane associated, that Yeh1 and Tgl1 localize to lipid particles, and that Yeh2 is enriched at the plasma membrane.

**A lipase triple mutant has no detectable steryl ester hydrolase activity in vitro.** To examine the contribution of the three lipases to steryl ester hydrolysis in vitro, we first determined whether the activity is present in a soluble or membrane-associated form. Therefore, wild-type cells were broken and the homogenate was separated into a soluble fraction and a membrane pellet. Membrane proteins were solubilized with 1% Triton X-100, and the steryl ester hydrolase activity in these fractions was determined by an in vitro assay with cholesterol–[1-$^{14}$C]oleate as the substrate (46). This analysis revealed that the steryl ester-hydrolyzing activity was detectable in the membrane fraction only, which is consistent with the localization of their tagged versions.

We next determined the steryl ester hydrolase activity in the detergent-solubilized membrane fraction from lipase single-, double-, and triple-mutant cells. This analysis revealed that wild-type cells harbored the highest specific cholesteryl esterase activity whereas the triple mutant was devoid of any detectable activity. The activity present in the single and double mutants indicates that Yeh2 provides the main activity in this in vitro assay, as the activity was reduced to nondetectable levels in strains that lack *YEH2* (Fig. 6A).

Since Yeh1 and Tgl1 are localized to lipid particles, we determined whether cholesteryl esterase activity is present in isolated lipid particles. Consistent with a lipid particle localization for two of these lipases, the specific cholesteryl esterase activity was ~24-fold enriched in the lipid particle fraction from wild-type cells. Analyses of the lipid particle-associated activity from the *yeh1*Δ and *tgl1*Δ single-mutant and *yeh1*Δ *tgl1*Δ double-mutant strains indicate that the detectable activity depends on Tgl1. The in vitro assay hence monitors the activity of Yeh2 and Tgl1 but does not detect any Yeh1-dependent esterase activity (Fig. 6B).

To determine whether the Yeh2-dependent activity is indeed enriched in the plasma membrane, as predicted on the basis of its localization, the cholesteryl esterase activity in plasma membranes from wild-type and *yeh2*Δ mutant cells was determined. Consistent with its localization and its high relative contribution to the total cellular activity, the Yeh2-specific activity was ~10-fold enriched in the plasma membrane fraction and this activity was completely dependent on *YEH2* (Fig. 6C). The results of these in vitro assays are thus consistent with the protein localization data. They also indicate that Yeh1 may require different conditions for its activity in vitro.

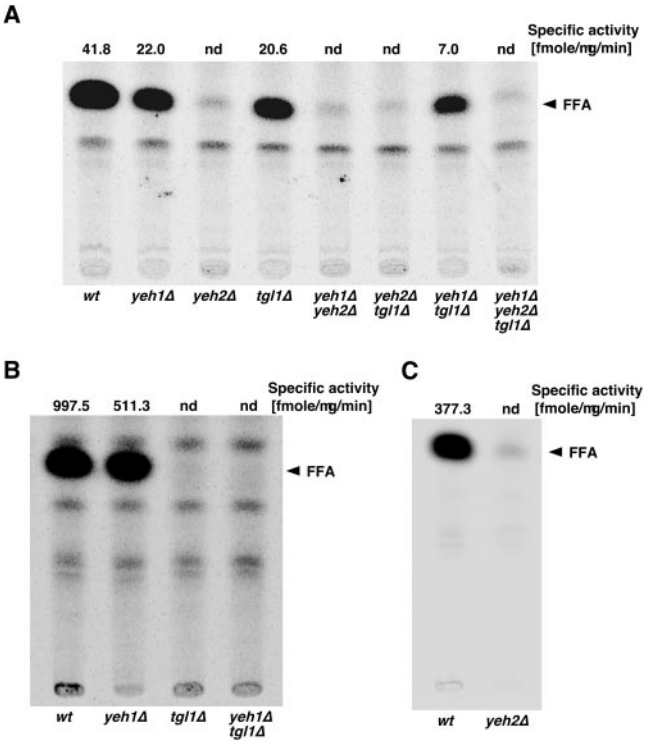



FIG. 6. Lipase triple-mutant cells lack steryl ester hydrolase activity in vitro. (A) In vitro steryl ester hydrolase activity in lipase single-, double-, and triple-mutant cells. Wild-type (*wt*; BY4742, YRS1533) and lipase single (*yeh1*Δ, YRS1972; *yeh2*Δ, YRS1971; *tgl1*Δ, YRS1973)-, double (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948)-, and triple (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840)-mutant cells were grown in YPD medium and broken with glass beads, and membranes were pelleted. The membrane pellet was solubilized with Triton X-100, and steryl ester hydrolase activity in the detergent extract was determined with cholesterol–[1-$^{14}$C]oleate as the substrate. The reaction was terminated after 60 min at 30°C, lipids were extracted and separated by TLC, and the released [$^{14}$C]oleic acid was quantified by radioscanning. The position of the liberated oleic acid is indicated (FFA). The specific activity of steryl ester hydrolase is indicated above the TLC lanes. nd, not detectable. (B) Tgl1-dependent steryl ester hydrolase activity is enriched in lipid particles. Lipid particles from cells of the indicated genotype were isolated, and the steryl ester hydrolase activity was determined in vitro. (C) Yeh2-dependent activity is enriched in the plasma membrane. Plasma membrane from cells of the indicated genotype was isolated, and steryl ester hydrolase activity was determined in vitro.

**Overexpression of Yeh1, Yeh2, and Tgl1 confers steryl ester hydrolysis in vivo.** To determine whether independent expression of one of the three lipases is sufficient to induce steryl ester mobilization in vivo, expression of these genes was placed under the control of an inducible *GAL1* promoter in a double-mutant background, thus allowing us to monitor the activity of each one of the lipases in the absence of the other two. At the same time, the lipases were tagged at their N termini with GFP, allowing the detection of each of the lipases under repressing (glucose) or inducing (galactose) conditions. Western blot analysis revealed that the GFP-tagged lipases were strongly expressed in cells grown in galactose-containing medium, but the tagged lipases were nondetectable when cells were

grown in glucose-containing medium (Fig. 7A). Examination of the steryl ester pool in the lipase-expressing cells revealed that constitutive expression of each of the three lipases is sufficient to lower the steryl ester pool compared to that in a lipase triple-mutant strain (Fig. 7B). Cells cultivated in glucose, on the other hand, accumulated higher levels of steryl esters than did a corresponding wild-type strain. Levels of triacylglycerols, the second major neutral lipid, however, remained unaffected under these conditions (Fig. 7C). These data thus indicate that the N-terminally GFP-tagged versions of the three lipases are functional and that expression of each one of the three lipases is sufficient to induce mobilization of steryl esters in vivo.

**Yeh1, Yeh2, and Tgl1 are integral membrane proteins.** To determine whether these lipases fulfill the biochemical properties of integral membrane proteins as predicted on the basis of their sequences and localization (Table 3; Fig. 4 and 5), we examined the solubility of their C-terminally GFP-tagged versions after extraction of membranes with salt (1 M NaCl), alkali (0.1 M $Na_2CO_3$), or detergent (1% Triton X-100 or 1% SDS). All three lipases remained membrane associated after salt or alkali treatment of membranes but were solubilized by detergents, indicating that they behave as integral membrane proteins (Fig. 8A).

To begin to understand the action of these enzymes on a mechanistic level, their membrane topology with regard to the localization of the active site was characterized in more detail. Therefore, the accessibility of the C-terminal GFP to cleavage by proteinase K was examined. Incubation of the 13,000 × *g* membrane pellet from strains expressing Yeh1-GFP or Tgl1-GFP with increasing concentrations of proteinase K revealed that the C termini of both proteins are readily degraded (Fig. 8B). Probing of these Western blots with an antibody against the luminal protein Kar2 indicates that the membrane seal remained intact during these incubations. These results thus indicate that the C termini of Yeh1 and Tgl1 are cytosolic.

To determine the accessibility of the C terminus of Yeh2, enriched plasma membrane vesicles from Yeh2-GFP-expressing cells were incubated with proteinase K. This resulted in the rapid appearance of a faster-migrating cleavage product that appeared to be the result of the removal of an ~17-kDa fragment from the N-terminal domain of Yeh2-GFP. Blotting of these membranes with an antibody against the GPI-anchored protein Gas1 revealed that Gas1 was protease protected, indicating that the enriched plasma membrane mainly consisted of inside-out vesicles and hence that the protected C terminus of Yeh2 is likely localized in a luminal-extracellular compartment.

To further refine this topological characterization, the accessibility of the N termini of the three lipases to protease was examined. Therefore, the lipases were tagged with GFP at their N termini and their expression was placed under the control of the *GAL1* promoter. Microscopic examination of cells expressing the N-terminally GFP-tagged lipases after overnight growth in galactose-containing medium revealed lipid particle staining for both GFP-Yeh1 and GFP-Tgl1 and plasma membrane staining for GFP-Yeh2 (data not shown). Proteinase accessibility of the N-terminal GFP was determined with the 13,000 × *g* membrane pellet from GFP-Yeh1- and GFP-Tgl1-expressing cells. These analyses revealed that the N-terminal epitope of Yeh1 was rapidly removed by the protease (Fig. 8C). Protease

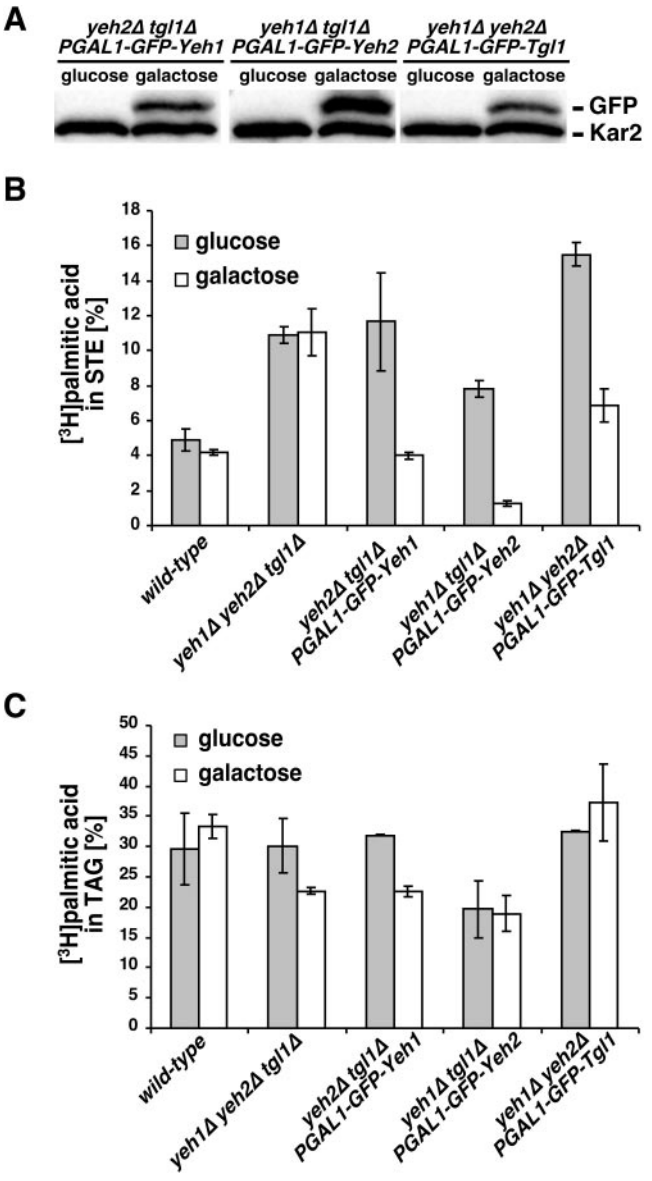



FIG. 7. Overexpression of N-terminally tagged lipases is sufficient to reduce steryl ester levels. (A) Overexpression of the lipases in a lipase double-mutant background. Strains (YRS2161, YRS2162, and YRS2163) that express an N-terminally GFP-tagged lipase under the control of a galactose-inducible promoter in the absence of the other two lipases were cultivated in either glucose- or galactose-containing rich medium for 16 h. Proteins were extracted, and equal amounts were subjected to Western blot analysis with an antibody against GFP and ER luminal Kar2. (B) Overexpression of GFP-Yeh1, GFP-Yeh2, and GFP-Tgl1 reduces steryl ester but not triacylglycerol levels in vivo. Cells of the indicated genotype were cultivated in either glucose- or galactose-containing rich medium and labeled with [$^3$H]palmitic acid for 16 h. The relative proportions of [$^3$H]palmitic acid in the steryl ester (STE) (B) and triacylglycerol (TAG) (C) pools were quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments.

cleavage of GFP-Tgl1, on the other hand, resulted in the rapid appearance of a faster-migrating product, indicating that the N terminus of GFP-Tgl1 is protected but that its C terminus is cleaved to release a fragment of ~12 kDa. Accessibility of the N terminus of Yeh2 was assessed again with enriched plasma

TABLE 3. Characteristics of the three predicted lipases

| Lipase | Molecular mass (kDa) | Signal sequence[a] | Signal sequence[b] | TMD[c] | TMD[d] | TMD[e] | Potential N-glycosylation site (position)[f] |
|---|---|---|---|---|---|---|---|
| Yeh1 | 66.51 | 31 | 32 | 8–36<br>392–415 | 13–32<br>342–363<br>390–412 | 12–36 | 139<br>140<br>419 |
| Yeh2 | 62.45 | | Signal anchor | 11–35<br>361–389 | 12–31<br>364–385 | 11–31<br>361–379<br>399–416 | 105 |
| Tgl1 | 62.98 | 28 | None | 4–32<br>243–271<br>354–381 | 11–30<br>195–213<br>249–268 | 14–33 | 46<br>424<br>536 |

[a] The signal sequences shown are indicated by the yeast genome database (http://db.yeastgenome.org) and were determined by using the application sigcleave available in the EMBOSS package (http://emboss.sourceforge.net/).
[b] Signal sequence derived by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).
[c] Transmembrane domains (TMDs) indicated by the yeast genome database on the basis of tmap applications available in the EMBOSS package.
[d] TMDs derived by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).
[e] TMDs derived by Phobius (http://phobius.cgb.ki.se/).
[f] The numbers and amino acid positions of potential N-linked glycosylation sites were predicted by using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

membrane vesicles. Incubation of the plasma membrane from GFP-Yeh2-expressing cells with protease resulted in rapid disappearance of GFP-Yeh2, indicating that the N terminus of Yeh2 is cytosolic.

To determine whether the potential N-glycosylation sites of the three lipases are accessible for ER luminal glycosylation, the C-terminally GFP-tagged versions of these proteins were subjected to treatment with endoglycosidase H and their electrophoretic mobility was compared with that of nontreated controls. The results of this analysis did not reveal any difference between treated and untreated samples, indicating that these proteins are not N-glycosylated and hence that the corresponding asparagine residues are not exposed to the ER lumen. Probing the same Western membranes with an antibody against a protein known to be N-glycosylated, Gpi8, indicated that the endoglycosidase H treatment was successful (data not shown).

Taken together, these results indicate that Yeh1 is a membrane protein that localizes to intracellular lipid particles with both N and C termini exposed to the cytosol, which suggests that the protein is anchored in the membrane by at least two transmembrane domains. Because lipases contain an alpha/beta hydrolase fold, whereas known integral membrane proteins contain either alpha-helical or beta-barrel structures in their membrane-embedded domain, we propose that Yeh1 has a hairpin-like transmembrane domain in its N-terminal region that anchors the protein to the membrane. This would allow both termini of Yeh1 to face the same compartment and would allow proper folding of the lipase domain. A hairpin-like membrane anchor is supported by the fact that one of the algorithms used to predict transmembrane regions, Phobius, indicates that the N terminus of Yeh1 has a particularly long hydrophobic stretch encompassing amino acids 12 to 36 (Table 3). A similar hairpin-like topology has been suggested for caveolin (18); in this case, Phobius predicts a membrane anchor of 23 amino acids, which is close to the 24 residues predicted for Yeh1.

Tgl1, which also localizes to lipid particles, has its C terminus exposed to the cytosol and the N terminus protected in a lumen compartment. Such a topology could be due to either one or three transmembrane domains. As the topology with three transmembrane domains would place the hydrolase domain into a transmembrane segment, we propose a type I topology for Tgl1 as the more plausible alternative. Yeh2 also has its N and C termini at different sites of the membrane, compatible with either one or three transmembrane domains. On the basis of the same arguments, we propose the simpler type II topology for Yeh2. A schematic overview of the proposed membrane topology of the three proteins is shown in Fig. 9. All three topologies are compatible with a potential alpha/beta hydrolase fold of the respective lipase.

## DISCUSSION

As an important step toward understanding sterol homeostasis at the cellular level, the aim of this study was to identify the steryl ester-hydrolyzing enzyme(s) of yeast. By a candidate gene approach, we identified a family of potential lipases with a predicted alpha/beta hydrolase-associated lipase domain as being required for steryl ester hydrolysis in vivo and in vitro.

The three lipases encoded by *YEH1*, *YEH2*, and *TGL1* constitute the yeast paralogues of the mammalian acid lipase family, which includes lysosomal acid lipase A (LIPA), the enteric gastric/lingual lipase LIPF, and four novel members that are predicted on the basis of the human genome sequence, LIPL1 to LIPL4 (3, 5). The lysosomal acid lipase is a key enzyme in the intracellular degradation of neutral lipids that have been internalized through receptor-mediated endocytosis of lipoprotein particles (19). The enzyme hydrolyzes cholesteryl esters and triacylglycerols, releasing free cholesterol and fatty acids. The released cholesterol rapidly equilibrates with the cellular pool to regulate its endogenous synthesis, esterification, and receptor-mediated uptake (11). Feedback regulation of sterol synthesis by the steryl ester pool in yeast is poorly characterized, but the lipase mutants now provide an important tool to address this pathway genetically. LIPA is active toward both steryl esters and triacylglycerols (5); the yeast enzymes, on the other hand, appear to be more specific for steryl esters, as triacylglycerol mobilization in vivo is not affected in triple-mutant cells.

LIPA deficiency in humans causes two rare autosomal re-

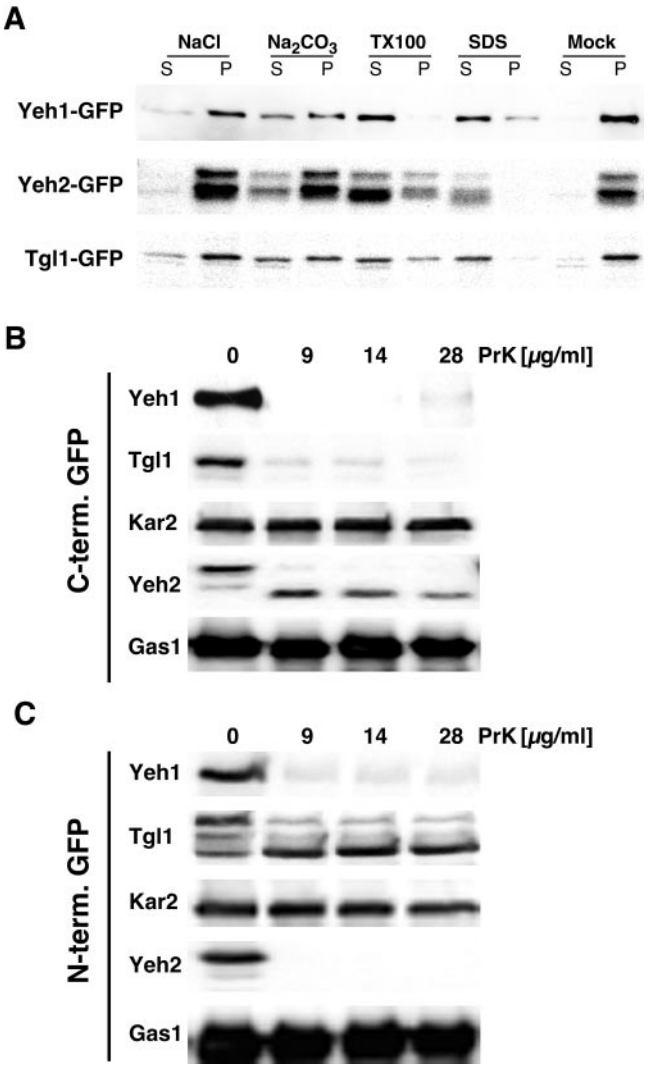



FIG. 8. Yeh1, Yeh2, and Tgl1 are integral membrane proteins. (A) Yeh1-, Yeh2-, and Tgl1-GFP are solubilized by detergents. Strains expressing the C-terminally (C-term.) tagged lipases (Yeh1-GFP, YRS1974; Yeh2-GFP, YRS2986; Tgl1-GFP, YRS1858) were grown in YPD medium, cells were broken with glass beads, and membranes were pelleted by centrifugation at 13,000 × *g*. Fifty micrograms of protein from the membrane pellet was incubated for 30 min at 4°C with 1 M NaCl, 0.1 M $Na_2CO_3$, 1% Triton X-100 (TX100), 1% SDS, or buffer alone (Mock) and then centrifuged at 13,000 × *g* for 30 min to yield soluble (S) and pellet (P) fractions. Proteins were precipitated by TCA, and 10 μg was separated by electrophoresis and probed for the presence of the GFP epitope with an anti-GFP antibody. (B) Protease sensitivity of the C-terminal GFP on Yeh1, Yeh2, and Tgl1. Fifty micrograms of protein from either the 13,000 × *g* membrane pellet (Yeh1-GFP and Tgl1-GFP) or the enriched plasma membrane fraction (Yeh2-GFP) was incubated with the indicated proteinase K (PrK) concentration for 30 min on ice. Proteins were precipitated by TCA, and 10 μg was separated by electrophoresis and probed for the presence of the GFP epitope or for Kar2 and Gas1, respectively. (C) Protease sensitivity of the N-terminal (N-term.) GFP on Yeh1, Yeh2, and Tgl1. Strains expressing the N-terminally tagged lipases (GFP-Yeh1, YRS2083; GFP-Yeh2, YRS2085; GFP-Tgl1, YRS2084) were grown in YPGal medium, cells were broken with glass beads, and proteins from either the 13,000 × *g* membrane pellet (GFP-Yeh1 and GFP-Tgl1) or the enriched plasma membrane fraction (GFP-Yeh2) were incubated with the indicated proteinase K concentration for 30 min on ice. Proteins were precipitated by TCA, separated by electrophoresis, and probed for the presence of the GFP epitope and for Kar2 and Gas1, respectively.

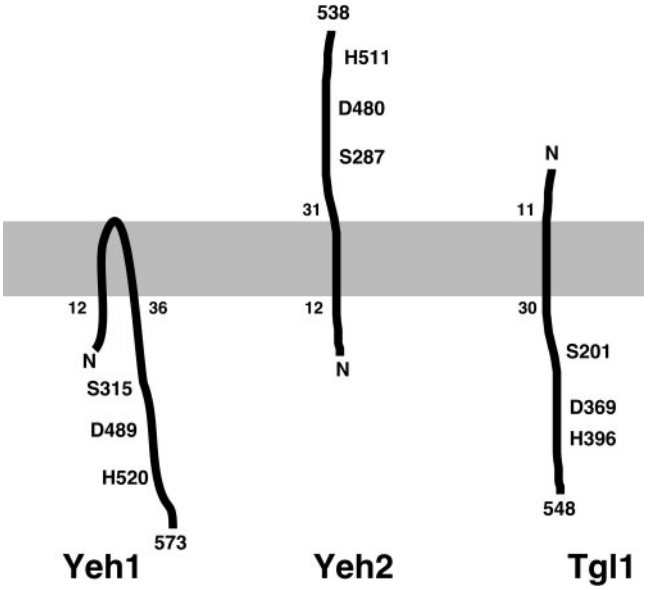



FIG. 9. Schematic representation of the proposed membrane topology of yeast lipases Yeh1, Yeh2, and Tgl1 with regard to the catalytic-triad residues.

cessive disorders, Wolman disease and cholesterol ester storage disease (4, 6, 13, 41). Wolman disease is lethal within the first year of life because of hepatosplenomegaly, adrenal calcification, and massive accumulation of triglycerides and cholesteryl esters in these organs, as well as in macrophages and blood vessels. Cholesterol ester storage disease is a less severe disorder with longer survival, hepatomegaly, premature atherosclerosis, and dyslipoproteinemias. Patients with the milder form of this lipid storage disease retain some residual LIPA activity (39). The crucial role of LIPA in steryl ester metabolism also prompted its consideration as an important factor in atherogenesis (6).

LIPA localizes to endosomes, where it cleaves low-density-lipoprotein-derived substrates. Our subcellular localization of GFP-tagged versions of the three yeast lipases indicates that Yeh1 and Tgl1 both localize to lipid particles whereas Yeh2 is enriched in the plasma membrane. Lipid particle localization of two of the three yeast steryl ester hydrolases, Yeh1 and Tgl1, is likely to allow regulated access to their neutral lipid substrates, which are stored in these particles (29). Intriguingly, however, both lipases appear to be membrane proteins on the basis of the detergent requirement for their solubilization. Yeh1 and Tgl1 are each proposed to be an integral membrane protein with an N-terminal transmembrane segment(s). This membrane anchoring is particularly remarkable in light of the fact that the lipid particle membrane has been proposed to be a monolayer rather than a normal bilayer membrane (29). It would thus be interesting to examine how these transmembrane domains can be accommodated in a lipid monolayer. Alternatively, it might be conceivable that the two lipases localize to domains of the lipid particle membrane that retain a bilayer rather than a monolayer structure.

The role of Yeh2 in light of its plasma membrane localization is less obvious than that of lipid particle-localized Yeh1 and Tgl1. But given the fact that the active site of the enzyme

is located in a luminal-extracellular compartment, it is tempting to speculate that Yeh2 may be important to cleave either extracellular or endocytosed steryl esters. While yeast readily takes up exogenous fatty acids, sterols are taken up only under anaerobic conditions (32). The observation that Yeh2 alone in a *yeh1*Δ *tgl1*Δ double mutant does not efficiently mobilize steryl esters in vivo is consistent with a limiting access of the enzyme to the intracellular steryl ester pool, as predicted on the basis of the localization and topology of the enzyme. It is interesting that Yeh2 binds phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-triphosphate in vitro (54). While phosphatidylinositol-3,4,5-triphosphate has not been detected in *S. cerevisiae* (35, 36), it remains to be seen whether these signaling lipids affect the activity of the enzyme. The size variants of Yeh2 detected on Western blots are likely to be due to O-linked glycosylation of the luminal domain of Yeh2, which would be consistent with the proposed type II topology of the enzyme.

Levels of steryl esters are coordinated with the growth phase (9, 48). It is thus necessary that their synthesis, mobilization, or both processes are tightly regulated. An interesting question for further studies will thus be to examine whether and, if so, how the lipases obtain regulated access to their substrates and to further investigate the molecular mechanism of this regulation. Mobilization of steryl esters from lipid particles requires energy and ongoing protein synthesis but is independent of microtubules (28). Recent results obtained with mammalian cells suggest that an interplay between components that localize to the lipid droplets, such as perilipin and the lipase, in this case HSL, is important to coordinate substrate access. This interplay is regulated by protein kinase A to increase the rate of lipolysis by 30- to 100-fold (44). Even though yeast lipid particles lack any obvious perilipin orthologue, it is interesting that both Yeh1 and Tgl1, but not Yeh2, contain potential cyclic-AMP-dependent protein kinase A phosphorylation sites. Taken together, the identification and topological characterization of three yeast lipases now provide the opportunity to use *S. cerevisiae* to further characterize the molecular mechanisms that control sterol homeostasis at the cellular level.

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