

Yeh1 Constitutes the Major Steryl Ester Hydrolase under Heme-Deficient Conditions in *Saccharomyces cerevisiae*

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Steryl esters are stored in intracellular lipid droplets from which they are mobilized upon demand and hydrolyzed to yield free sterols and fatty acids. The mechanisms that control steryl ester mobilization are not well understood. We have previously identified a family of three lipases of *Saccharomyces cerevisiae* that are required for efficient steryl ester hydrolysis, Yeh1, Yeh2, and Tgl1 (R. Köffel, R. Tiwari, L. Falquet, and R. Schneider, Mol. Cell. Biol. 25:1655–1668, 2005). Both Yeh1 and Tgl1 localize to lipid droplets, whereas Yeh2 is localized to the plasma membrane. To characterize the precise function of these three partially redundant lipases, we examined steryl ester mobilization under heme-deficient conditions. *S. cerevisiae* is a facultative anaerobic organism that becomes auxotrophic for sterols and unsaturated fatty acids in the absence of molecular oxygen. Anaerobic conditions can be mimicked in cells that are deficient for heme synthesis. We here report that Yeh1 is the sole active steryl ester hydrolase under such heme-deficient conditions, indicating that Yeh1 is activated whereas Yeh2 and Tgl1 are inactivated by the lack of heme. The heme-dependent activation of Yeh1 is mediated at least in part by an increase in steady-state levels of Yeh1 at the expense of Yeh2 and Tgl1 in exponentially growing cells. This increase in steady-state levels of Yeh1 requires Rox3, a component of the mediator complex that regulates transcription by RNA polymerase II. These data thus provide the first link between fat degradation and the transcriptional control of lipase activity in yeast.

Sterols are essential lipids of eukaryotic cells, where they occur in two major forms: free sterols and steryl esters. Free sterols are synthesized in the endoplasmic reticulum (ER) membrane, and they are greatly enriched at the plasma membrane, which harbors 90% of the free sterol pool of a cell (13). Steryl esters, on the other hand, serve to store fatty acids and sterols for energy production and membrane synthesis. These nonmembrane-forming neutral lipids are deposited in intracellular lipid droplets. The conversion of free sterols and acyl coenzyme A's to steryl esters is catalyzed by acyl coenzyme A:sterol acyltransferases (ACATs) that are localized in the ER membrane (3). The formation and hydrolysis of steryl esters are important in maintaining sterol homeostasis, as the steryl ester pool conceptually serves to buffer both excess and a lack of free sterols (2).

Saccharomyces cerevisiae harbors two ACAT genes, *ARE1* and *ARE2*. Deletion of both genes results in the absence of steryl esters but does not compromise mitotic growth, indicating that synthesis of steryl esters is not essential under standard growth conditions (27, 28). Are2 provides the major activity to esterify the mature sterol of yeast, ergosterol, under aerobic conditions, and its activity is important to complete meiosis, as an *are2*Δ homozygous diploid has a reduced sporulation efficiency and arrests after the first meiotic division (28). Are1, on the other hand, preferentially esterifies sterol intermediates and is upregulated under heme-deficient conditions (10, 24, 25, 30). These observations indicate that Are1 is physiologically important under

anaerobic conditions, when heme is limiting and sterol precursors accumulate.

Steryl esters synthesized by Are1 and Are2 are stored in intracellular lipid droplets from which they can be remobilized upon demand and are then hydrolyzed by the action of three steryl ester hydrolases, Yeh1, Yeh2, and Tgl1 (8, 11, 14, 17). Yeh1 and Tgl1 localize to lipid particles, whereas Yeh2 is enriched at the plasma membrane (8, 11, 17). All three lipases are membrane anchored, and a triple mutant strain lacking all three lipases lacks steryl ester hydrolysis *in vivo*, indicating that together Yeh1, Yeh2, and Tgl1 account for all the steryl ester hydrolase activity that is present in yeast (11). The fact that the lipase triple mutant is viable furthermore indicates that steryl ester hydrolysis is not essential for mitotic growth (11).

The aim of this study was to characterize the role of the three lipases in steryl ester hydrolysis in more detail and to begin to dissect the apparent functional redundancy of these lipases. We here concentrate on the role of the three lipases under heme-deficient conditions and show that under these conditions, Yeh2 and Tgl1 are inactive *in vivo* and that all the steryl ester hydrolase activity present in heme-deficient cells is attributed to Yeh1. Activation of Yeh1 under heme deficiency is accompanied by an upregulation of steady-state levels of Yeh1 at the expense of Tgl1 and Yeh2 in exponentially growing cells. The heme-dependent inactivation of Yeh2 and Tgl1 is overcome by overexpression of the enzymes, indicating that expression of Yeh2 and Tgl1 is rate limiting under heme deficiency. Activation of Yeh1 under these conditions requires Rox3, a component of the mediator complex that controls RNA polymerase II activity, indicating that steryl ester degradation is controlled at least in part by transcriptional regulation of *YEH1* expression (6, 18).

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MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast strains used in this study are listed in Table 1. Strains bearing single deletions of nonessential genes were obtained from EUROSCARF (see <http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html> [26]). Strains were cultivated in YPD-rich media (1% Bacto yeast extract, 2% Bacto peptone [USBiological, Swampscott, MA], 2% dextrose) or minimal media. Media supplemented with sterols and fatty acids contained 5 mg/ml Tween 80 and 20 μ g/ml ergosterol, cholesterol, or lanosterol (Sigma Chemical Co., St Louis, MO). *hem1* Δ mutant cells were supplemented with 20 μ g/ml delta-aminolevulinic acid (ALA). Selection for the *kanMX4* marker was on media containing 200 μ g/ml G418 (Gibco-BRL, Life Technologies). Double and triple mutant strains were generated by crossing of single mutants and by gene disruption, using the PCR deletion cassettes (15) and the gene-specific primers used for the initial characterization of the three lipases (11). The plasmid pHEM1-LEU2 containing the *hem1::LEU2* disruption cassette (kindly provided by I. Hapala, Slovak Academy of Sciences, Bratislava, Slovak Republic) was cut with BamHI/HindIII to release the disruption cassette, and yeast transformants were selected on minimal media without leucine but supplemented with ALA. Correct insertion of the disruption cassette at the *HEM1* locus was confirmed by phenotypic analysis of the transformants, i.e., growth on ALA-supplemented media but no growth on nonsupplemented media. Yeast was transformed by treatment with lithium acetate (7).

Western blot analysis. Protein concentrations were determined by the method of Lowry et al. (16), using the Folin reagent and bovine serum albumin as standards. Proteins were precipitated with 10% trichloroacetic acid, resuspended in sample buffer, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were probed with rabbit antisera against green fluorescent protein (GFP) (1:5,000; Torrey Pines Biolabs, Inc., Houston, TX), Kar2 (1:5,000; M. Rose, Princeton University, New Jersey), or Wbp1 (1:1,000; M. Aebi, ETH Zurich, Switzerland).

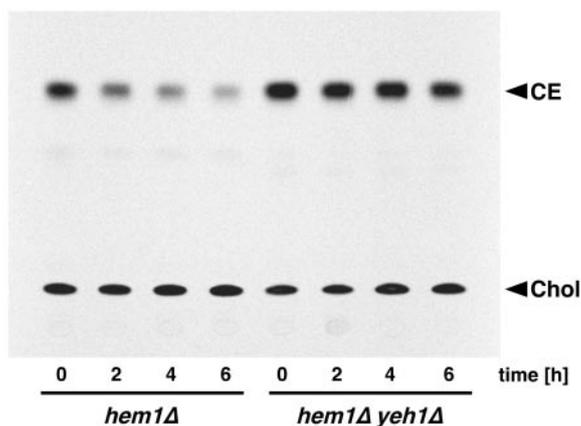
Fluorescence microscopy. In vivo localization of GFP-tagged versions of Yeh1, Yeh2, and Tgl1 in the heme-deficient background was performed by fluorescence microscopy, using a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software.

In vivo neutral lipid mobilization. The sterol ester pool was labeled by incubating the cells either with 0.025 μ Ci/ml [14 C]cholesterol or with 10 μ Ci/ml [3 H]palmitic acid (American Radiolabeled Chemicals Inc., St. Louis, MO) for 16 h at 24°C. Cells were then washed and diluted into liquid YPD media containing Tween 80 and either cholesterol or ergosterol and 30 μ g/ml terbinafine to block squalene epoxidase (9). The inclusion of terbinafine is not required to induce the mobilization of sterol esters but prevents the formation of lanosterol and thus more closely mimics an anaerobic block in sterol biosynthesis than it does heme deficiency alone. 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 dried. Lipids were separated on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) developed in petroleum ether-diethylether-acetic acid (70:30:2 [by volume]) 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 using a PhosphorImager (Bio-Rad Laboratories, Hercules, CA).

Overexpression of sterol ester hydrolases and quantification of the endogenous sterol ester pool. Heme-deficient strains were cultivated in cholesterol-containing media with either glucose (repressing conditions) or galactose (inducing conditions) as the carbon source. The neutral lipid pool was labeled to steady-state levels by incubating the cells with 0.025 μ Ci/ml [14 C]cholesterol for 16 h at 30°C. Cells were diluted into YPD or YP-galactose liquid media containing terbinafine (30 μ g/ml). Aliquots of cells were removed after 6 h of growth, and 3 optical density (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 dried, and lipids were separated on TLC plates developed in petroleum ether-diethylether-acetic acid (70:30:2 [by volume]) and quantified by scanning with a Berthold Tracemaster 40 automatic TLC linear analyzer.

Northern analysis. Total RNA was prepared by extraction of cells with hot acidic phenol. RNA (20 μ g) was separated on denaturing 1% agarose gels, transferred to nitrocellulose membranes (NEN GeneScreen Plus), and hybridized overnight with 32 P-labeled RNA probes made by random priming of gene-specific PCR products. Membranes were washed at high stringency and exposed to a PhosphorImager screen.

A



B

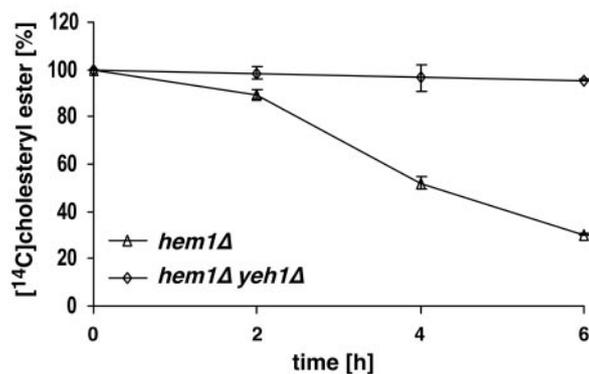


FIG. 1. *YEH1* is required for sterol ester mobilization in a heme-deficient background. (A) Heme-deficient wild-type (YRS1707) and *yeh1* Δ mutant (YRS1710) cells were labeled for 16 h with [14 C]cholesterol, and the kinetics of sterol ester mobilization in vivo was analyzed by determining sterol ester levels at 0, 2, 4, and 6 h after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, as described in Materials and Methods. Chol, cholesterol; CE, cholesteryl esters. (B) Levels of [14 C]cholesteryl esters were quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations from two independent experiments.

RESULTS

***YEH1* is required for efficient in vivo mobilization of sterol esters in heme-deficient cells.** Given that sterol ester synthesis is differentially regulated by the availability of oxygen, we wondered whether the same may also hold true for sterol ester hydrolysis. Anaerobiosis can be mimicked by heme deficiency, which renders the cells auxotrophic for methionine, sterols, and unsaturated fatty acids, because the synthesis of these compounds require cytochromes and molecular oxygen (5). To examine the rate of sterol ester hydrolysis in heme-deficient sterol ester hydrolase mutants, cells were labeled with [14 C]cholesterol and then diluted into fresh media containing cold cholesterol and Tween 80 as a source for unsaturated fatty acids. Samples were then withdrawn after 0, 2, 4, and 6 h of growth, lipids were isolated and separated by TLC, and the level of [14 C]cholesterol-labeled sterol esters was quantified by radio scanning of TLC plates. Under these conditions, the

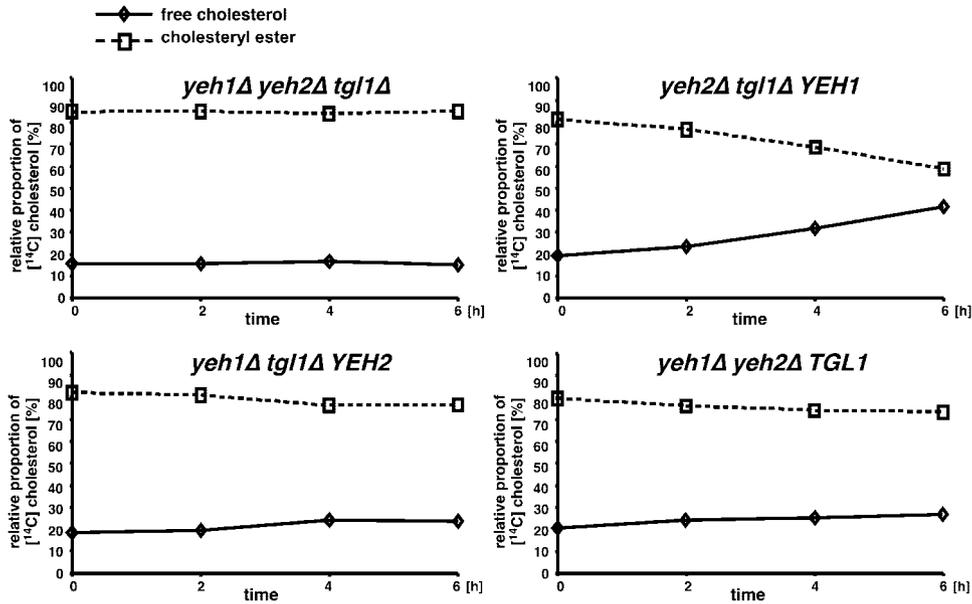


FIG. 2. *YEH2* and *TGL1* do not contribute to sterol ester hydrolysis under heme-deficient conditions. Heme-deficient lipase triple (*yeh1Δ yeh2Δ tgl1Δ*, YRS1922) and double (*yeh1Δ tgl1Δ*, YRS1923; *yeh2Δ tgl1Δ*, YRS1961; and *yeh1Δ yeh2Δ*, YRS2045) mutant cells were labeled for 16 h with [¹⁴C]cholesterol, and the kinetics of sterol ester mobilization in vivo was analyzed after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, as described in Materials and Methods. Levels of free and esterified [¹⁴C]cholesterol were quantified by radioscanning of TLC plates. Data shown are representative of two independent experiments, with standard deviations of less than 5% between experiments.

heme-deficient wild-type strain efficiently mobilizes sterol esters, resulting in an approximately threefold drop of the sterol ester pool over a 6-h period. Cells lacking *YEH1*, on the other hand, maintained most of their sterol ester pool, indicating that *Yeh1* is required for efficient hydrolysis of sterol esters under heme-deficient conditions (Fig. 1). Absence of either *YEH2* or *TGL1*, on the other hand, did not affect the rate of sterol ester mobilization compared to wild-type cells, indicating that *Yeh1* may be the only sterol ester hydrolase that is active under heme deficiency (data not shown).

***YEH1* is the only sterol ester hydrolase gene active under heme deficiency.** To examine the contribution of *YEH1*, *YEH2*, or *TGL1* to sterol ester hydrolysis in the absence of the other two hydrolases, we examined sterol ester mobilization in the lipase triple mutant and the three lipase double mutant combinations. Therefore, heme-deficient sterol ester hydrolase triple and double mutant strains were generated and cells were labeled with [¹⁴C]cholesterol. The cells were again diluted into fresh media containing cold cholesterol, and the mobilization of the preexisting sterol ester pool was examined over time.

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

Strain	Relevant genotype	Source and/or reference
YRS1533	<i>BY4742; MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0</i>	EUROSCARF; 26
YRS1707	<i>BY4742; MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 hem1::LEU2</i>	This study
YRS1710	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1::kanMX4 hem1::LEU2</i>	This study
YRS1709	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 tgl1::kanMX4 hem1::LEU2</i>	This study
YRS1708	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh2::kanMX4 hem1::LEU2</i>	This study
YRS1922	<i>MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1::HIS3MX6 yeh2::kanMX4 tgl1::kanMX4 hem1::LEU2</i>	This study
YRS2045	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1::HIS3MX6 yeh2::kanMX4 hem1::LEU2</i>	This study
YRS1923	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1::HIS3MX6 tgl1::kanMX4 hem1::LEU2</i>	This study
YRS1961	<i>MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2::kanMX4 tgl1::kanMX4 hem1::LEU2</i>	This study
YRS2046	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 YEH1-GFP-HIS3MX6 hem1::LEU2</i>	This study
YRS2048	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 YEH2-GFP-HIS3MX6 hem1::LEU2</i>	This study
YRS2047	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 TGL1-GFP-HIS3MX6 hem1::LEU2</i>	This study
YRS2184	<i>MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2::kanMX4 tgl1::kanMX4 trp1 TRP1-GAL1-GFP-YEH1 hem1::LEU2</i>	This study
YRS2183	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1 yeh1::HIS3MX6 tgl1::kanMX4 TRP1-GAL1-GFP-YEH2 hem1::LEU2</i>	This study
YRS2182	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1 yeh1::HIS3MX6 yeh2::kanMX4 TRP1-GAL1-GFP-TGL1 hem1::LEU2</i>	This study
YRS1766	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 rox3::kanMX4 hem1::LEU2</i>	19
YRS2740	<i>MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 rox3::kanMX4 YEH1-GFP-HIS3MX6 hem1::LEU2</i>	This study

This analysis revealed that only cells with a functional *YEH1* were able to mobilize steryl esters, indicating that Yeh1 is the only steryl ester hydrolase that is active under heme-deficient conditions and that in the absence of *YEH1*, neither Yeh2 nor Tgl1 can replace Yeh1 (Fig. 2). In the absence of either Tgl1 or Yeh2 or both lipases, however, Yeh1p-dependent steryl ester hydrolysis is approximately fourfold less efficient than in wild-type cells, indicating that the presence of either Tgl1 or Yeh2 directly or indirectly increases the rate of steryl ester hydrolysis.

Yeh1 is active against different steryl ester substrates. Oxygen-limiting conditions result in the accumulation of intermediates of the sterol biosynthetic pathway, particularly lanosterol, as many of the biosynthetic steps along this pathway are oxygen and/or heme dependent (4, 5, 20). These sterol intermediates are then preferentially esterified by Are1, which provides the major ACAT activity under heme-deficient or anaerobic conditions (10, 24). To examine whether the activation of Yeh1 at the expense of Yeh2 and Tgl1 under heme-deficient conditions is due to a possible substrate preference of the enzyme for nonergosterol-containing steryl esters, we examined the apparent *in vivo* substrate specificity of Yeh1 towards different steryl esters. Therefore, heme-deficient cells were precultivated in media containing ergosterol, cholesterol, or lanosterol; they were then labeled with [³H]palmitic acid to radiolabel the steryl ester pool and shifted to fresh media containing the type of sterol with which the cells were precultivated, and steryl ester mobilization was analyzed over time. This analysis revealed that the turnover of the different types of sterols remained constant and was in each case dependent on Yeh1, indicating that the activity of Yeh1 under heme deficiency is not due to a substrate preference of the enzyme for nonergosterol-containing steryl esters. In addition, the analysis also revealed that the lack of activity of Yeh2 and Tgl1 under heme deficiency cannot be completely explained by a preference of these enzymes for ergosteryl esters, even though Yeh2 does appear to exhibit increased activity against ergosteryl esters (Fig. 3). Taken together, these observations indicate that the activities of the three lipases are differentially regulated by the heme status of the cells but are largely independent of the type of steryl ester present.

Heme deficiency results in increased steady-state levels of Yeh1. To begin to characterize the molecular mechanism(s) that is responsible for the high *in vivo* activity of Yeh1 and the apparent inactivity of Yeh2 and Tgl1 under heme deficiency, we first examined whether heme deficiency affects the subcellular distribution or steady-state levels of these three lipases. *HEM1* deficiency can be bypassed by supplementing the cells with the enzymatic product of the Hem1-catalyzed first step in heme biosynthesis, ALA (5). Thus, when supplemented with ALA, *hem1Δ* mutant cells have a normal aerobic metabolism and are capable of growing on nonfermentative carbon sources. To examine whether heme deficiency induces an altered subcellular localization or expression level of the lipases, heme-deficient cells expressing a functional, GFP-tagged version of the lipases were cultivated in media containing ALA or cholesterol plus Tween 80, and the subcellular localization of the lipases was examined by fluorescence microscopy. This analysis revealed that Yeh1 and Tgl1 localize to lipid particles under heme-proficient and heme-deficient conditions, indicating that these two lipases do not change their subcellular lo-

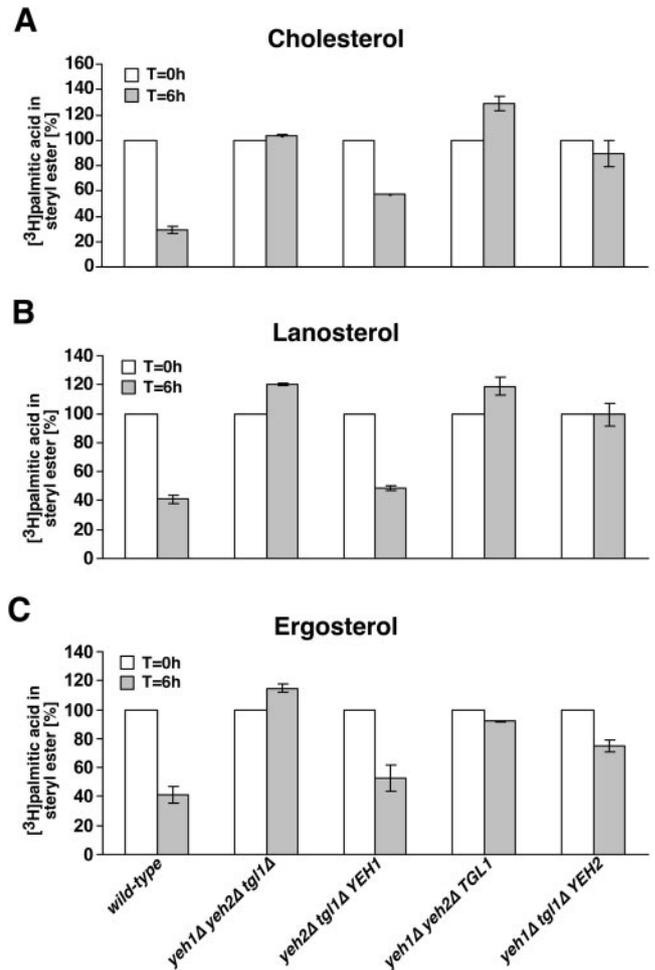


FIG. 3. Yeh1 is active against different steryl ester substrates. Heme-deficient wild-type (YRS1707), lipase triple mutant (YRS1922), and lipase double mutant (YRS1923, YRS1961, and YRS2045) cells were precultivated in media containing ergosterol, cholesterol, or lanosterol, and the steryl ester pool was labeled by incubating cells with [³H]palmitic acid for 16 h. Cells were then diluted into fresh media containing the same sterol as that used for the precultivation, and samples were removed after 0 h and 6 h of growth. Lipids were extracted and analyzed by TLC, and levels of [³H]palmitate in cholesteryl, lanosteryl, and ergosteryl esters were quantified by radioscaning. Values represent means and standard deviations from two independent experiments.

calization in response to heme status (Fig. 4A) (11). Yeh2, on the other hand, was previously localized to the plasma membrane in heme-proficient cells and displayed no alteration in the subcellular distribution upon heme depletion, again indicating that the subcellular localization of Yeh2 is not affected by the heme status of the cell (Fig. 4A) (11). The strong vacuolar staining observed here with the C-terminally GFP-tagged Yeh2 is likely due to vacuolar turnover of this fusion protein and is not observed with an N-terminal GFP fusion (11).

To examine whether heme depletion affects the steady-state levels of these lipases, strains expressing C-terminally GFP-tagged lipases from their native promoters were precultured in media supplemented with either ALA or cholesterol and diluted into fresh media containing the same supplements, and

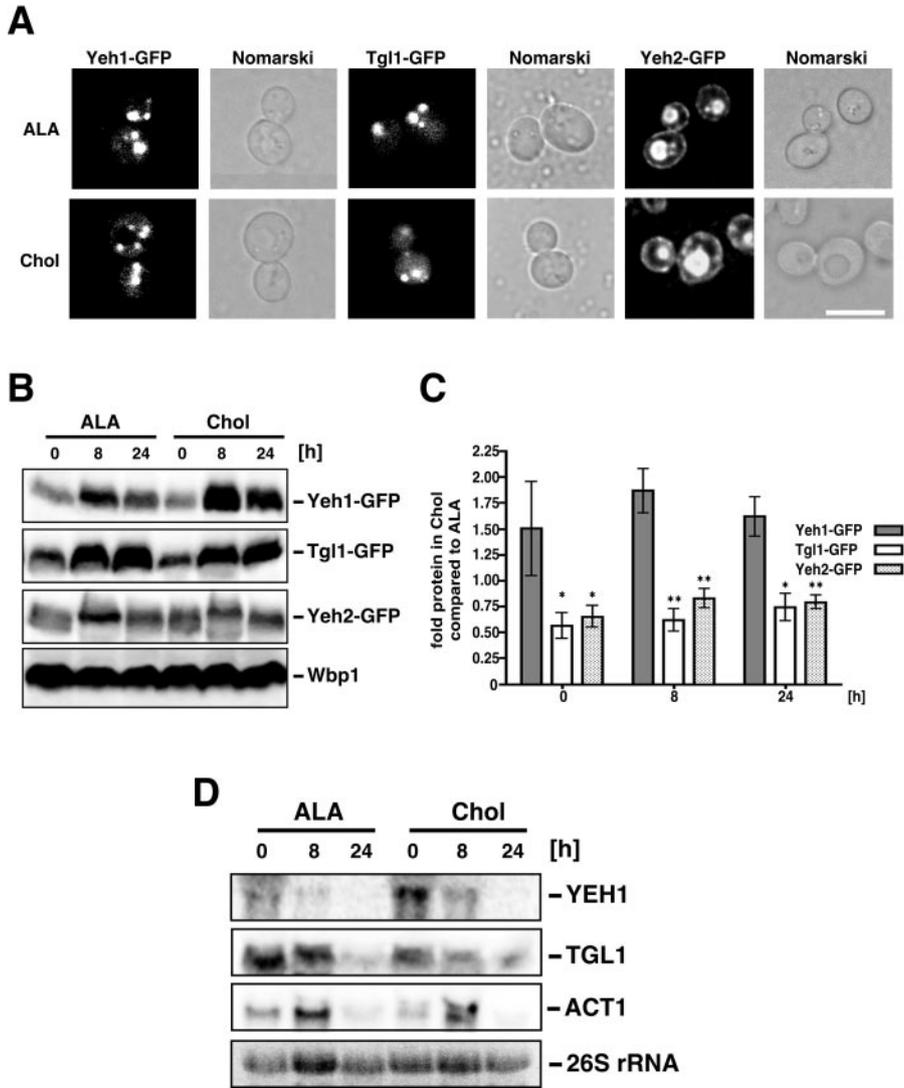


FIG. 4. Heme deficiency results in increased steady-state levels of Yeh1 at the expense of Yeh2 and Tgl1. (A) Heme-deficient cells expressing a GFP-tagged version of Yeh1 (YRS2046), Yeh2 (YRS2048), and Tgl1 (YRS2047) were cultivated in media containing either ALA or cholesterol (Chol) plus Tween 80 for 16 h, and the subcellular localization of the lipases was examined by fluorescence microscopy. Bar, 5 μ m. (B) Heme-deficient cells expressing GFP-tagged lipases were precultivated in media containing either ALA or cholesterol plus Tween 80 for 16 h and diluted to an OD₆₀₀ of 0.8, and samples were removed at the indicated time points. Steady-state levels of the GFP-tagged enzymes were analyzed by Western blotting, using Wbp1 as a loading control. (C) Signal intensities on Western blots were quantified by densitometry. Data represent means \pm standard errors of the means ($n = 3$). Significance of the difference between the steady-state levels of Yeh1-GFP and Tgl1-GFP and between Yeh1-GFP and Yeh2-GFP, as based on a two-tailed unpaired t test, is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$). (D) Northern analysis of transcript levels. Heme-deficient wild-type cells (YRS1707) were cultivated in media containing either ALA or cholesterol plus Tween 80 for 16 h and diluted to an OD₆₀₀ of 0.8, and samples were removed at the indicated time points. RNA was extracted, and transcript levels of *YEH1*, *TGL1*, and actin (*ACT1*) were determined by Northern blotting.

steady-state levels of the tagged lipases were examined by Western blotting at 0, 8, and 24 h after cell dilution. Protein loading was normalized to Wbp1, an ER-localized subunit of the oligosaccharyltransferase complex (Fig. 4B) (23). This analysis revealed that Yeh1 levels are approximately 1.5-fold higher in cholesterol-grown cells than in cells cultivated in ALA. Under these conditions, levels of Tgl1, on the other hand, are slightly decreased whereas those of Yeh2 remain constant. These data would thus indicate that the differential regulation of the in vivo activity of these three steryl ester hydrolases is mediated, at least in part, by an upregulation of

the steady-state levels of Yeh1. These differences in steady-state levels of the enzymes are not due to differences in the growth phase of the cells analyzed, as they grew with comparable rates. The observed upregulation of Yeh1, however, cannot account solely for the strong dependence of steryl ester hydrolysis on Yeh1, as both Tgl1 and Yeh2 are present and only slightly downregulated. Upregulation of Yeh1 levels under heme-deficient conditions is due largely to increased steady-state levels of *YEH1* transcripts as revealed by Northern analysis, indicating that Yeh1 expression is subject to heme-dependent regulation (Fig. 4D).

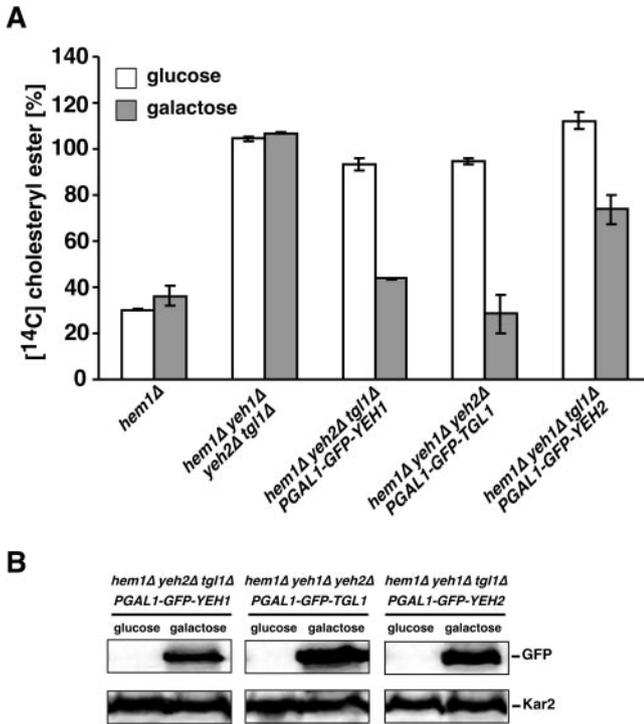


FIG. 5. The heme-dependent inactivation of Yeh2 and Tgl1 is overcome by overexpression of the enzymes. (A) Heme-deficient wild-type cells (YRS1707), *yeh1Δ yeh2Δ tgl1Δ* triple mutant cells (YRS1922), and cells expressing an N-terminally GFP-tagged version of Yeh1 (YRS2184), Yeh2 (YRS2183), and Tgl1 (YRS2182) from a regulatable *GAL1* promoter were labeled for 16 h with [¹⁴C]cholesterol, and the kinetics of steryl ester mobilization was analyzed after dilution of cells into fresh media containing either glucose or galactose. Cells were cultivated for 6 h, lipids were extracted and analyzed by TLC, and the relative content of [¹⁴C]cholesterol in the steryl ester pool was quantified by radioscanning of TLC plates. Values represent means and standard deviations from two independent experiments. (B) The level of repression or induction of the GFP-tagged lipases under the experimental conditions used for panel A was monitored by Western blot analysis, using Kar2 as a loading control.

Heme-dependent regulation of steryl ester hydrolysis is overcome by overexpression of Tgl1 and Yeh2. To test whether transcriptional regulation of the three lipases could account for the observed heme-dependent regulation, we examined whether overexpression of the lipases would overcome the apparent heme-dependent inactivation of Yeh2 and Tgl1. Therefore, expression of the three lipases was placed under the control of the inducible *GAL1* promoter. The *in vivo* activity of every one of the three lipases was then examined in the absence of the other two enzymes, i.e., in a lipase double mutant background. This analysis revealed that expression of each of the lipases is sufficient to induce steryl ester mobilization under heme-deficient conditions, as cells expressing the respective lipase display efficient mobilization of steryl esters when grown in galactose-containing media, i.e., under conditions where the promoter is active, but lack detectable steryl ester mobilization when cultivated in glucose-containing media, conditions that result in repression of the *GAL1* promoter (Fig. 5). These results thus indicate either that expression of Tgl1 and Yeh2 is rate limiting under heme-deficient conditions or that a possible

negative regulation at the posttranslational level is overcome by the strong overexpression of these enzymes.

Heme-dependent induction of Yeh1 requires ROX3. The expression of hypoxic genes under aerobic conditions is repressed by Rox1, whereas Hap1 positively regulates expression of normoxic genes (12; for a review, see reference 29). We have previously observed that mutants that lack Rox3, a component of the mediator complex that controls the activity of RNA polymerase II, have elevated levels of steryl esters under anaerobic conditions (6, 18, 19). We thus examined whether Rox3 is required for the induction of Yeh1 under heme deficiency. Therefore, heme-deficient *rox3Δ* mutant cells were labeled with [¹⁴C]cholesterol and mobilization of the radiolabeled steryl ester pool was examined over time. This analysis revealed that *rox3Δ* mutant cells were completely blocked in steryl ester mobilization (Fig. 6A). Analysis of the steady-state levels of Yeh1-GFP in the *rox3Δ* mutant background revealed that levels of this lipase are reduced under heme-deficient conditions, consistent with a requirement of Rox3 for a heme-dependent induction of Yeh1 (Fig. 6B).

Taken together, these data indicate that the heme-dependent activity of Yeh1 at the expense of Tgl1 and Yeh2 is mediated at least in part by upregulation of the steady-state levels of Yeh1 and a concomitant decrease of Tgl1 and Yeh2 in exponentially growing cells and that the mediator component Rox3 is required for this upregulation of Yeh1.

DISCUSSION

Levels of steryl esters are coordinated with the growth phase (1, 22). It is thus necessary that their synthesis or mobilization or both are tightly regulated. As an important step towards understanding sterol homeostasis at a cellular level, the aim of this study was to identify the *in vivo* role of the three partially redundant steryl ester hydrolases Yeh1, Yeh2, and Tgl1 under conditions that mimic anaerobiosis, i.e., heme deficiency. Our analysis indicates that under these conditions, Yeh1 is the major steryl ester hydrolase *in vivo* and that Tgl1 and Yeh2 do not significantly contribute to steryl ester mobilization in heme-deficient cells. Examination of the substrate specificities of the three lipases *in vivo* indicates that this differential regulation is not due to a preference of Yeh1 to hydrolyze steryl esters containing nonergosterol esters, which are known to accumulate under anaerobic or heme-deficient conditions and whose potential toxic effect is remedied by selective esterification by Are1, as Yeh1 is equally active against cholesteryl and lanosteryl esters as it is against ergosteryl esters (5, 10, 24).

Heme deficiency does not affect the membrane association, subcellular localization, or apparent molecular weight of Yeh1, Yeh2, and Tgl1 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GFP-tagged fusions (data not shown), suggesting that the heme-dependent activity of Yeh1 and apparent inactivity of Tgl1 and Yeh2 are not due to a relocalization of the enzymes. Heme deficiency, however, results in an increase in steady-state levels of Yeh1 and a concomitant downregulation of Tgl1 and Yeh2 in exponentially growing cells. Differential regulation of enzyme levels might thus at least in part explain the differential activation of Yeh1 under heme deficiency. The fact that Tgl1 and Yeh2 steady-state levels are not more strongly reduced under heme

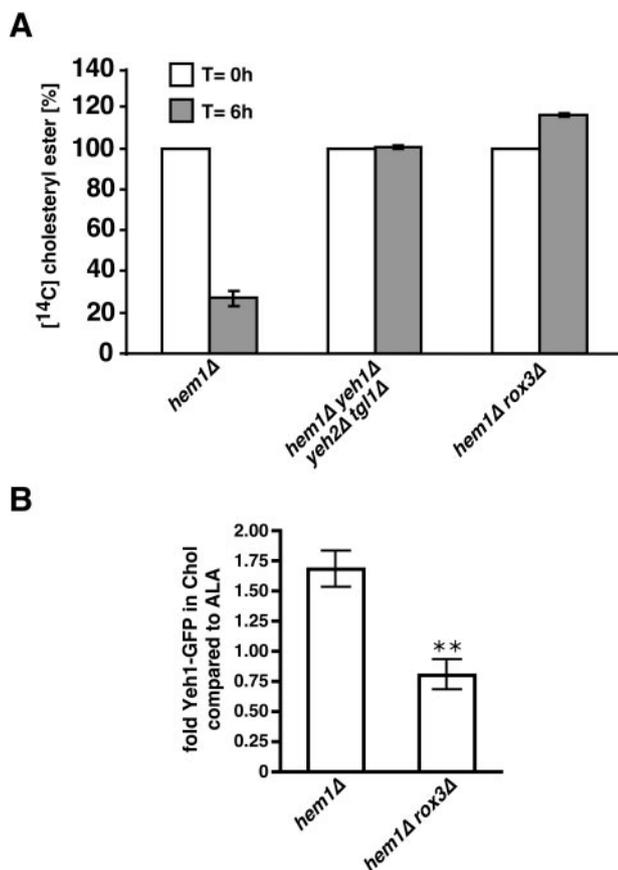


FIG. 6. *ROX3* is required for efficient mobilization of steryl esters under heme deficiency. (A) Heme-deficient wild-type (YRS1707), lipase triple mutant (YRS1922), and *rox3Δ* mutant (YRS1766) cells were labeled for 16 h with [¹⁴C]cholesterol, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0 and 6 h after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, and the relative content of [¹⁴C]cholesterol in the steryl ester pool was quantified by radioscanning of TLC plates. Values represent means and standard deviations from two independent experiments. (B) *ROX3* is required for induction of Yeh1-GFP in lipid-supplemented media. Heme-deficient wild-type cells (YRS2046) and *rox3Δ* mutant cells expressing Yeh1-GFP (YRS2740) were cultivated for 24 h in media containing either ALA or cholesterol plus Tween 80, and levels of Yeh1-GFP were determined by quantification of Western blots, using Wbp1 as a loading control. Signal intensities were quantified by densitometry. Data represent means ± standard errors of the means ($n = 4$). Significance of the difference between the steady-state levels of Yeh1-GFP in wild-type and *rox3Δ* mutant cells, as based on a two-tailed unpaired *t* test, is indicated by asterisks ($P = 0.0042$).

deficiency, however, would indicate that these enzymes are inactivated at the posttranslational level. Such an inactivation mechanism may be overcome by strong overexpression of the two enzymes, explaining why the overexpression of Tgl1 and Yeh2 from the *GAL1* promoter restores their activity in vivo. Cycloheximide chase experiments revealed that upregulation of Yeh1 under heme-deficient conditions does not affect the turnover of the enzyme, indicating that upregulation is due largely to increased expression of *YEH1* (data not shown). Consistent with such a transcriptional regulation of Yeh1, we find that Rox3, a component of the mediator complex that is

required for both activation and repression of RNA polymerase II activity, is required for steryl ester hydrolysis under heme deficiency and that cells lacking *ROX3* have reduced steady-state levels of Yeh1.

How exactly the lipases gain access to their substrates and how this step is regulated are not yet well understood. It is, however, interesting to note that under conditions where Tgl1 is inactive, the enzyme is still present and localized to lipid particles, suggesting that it lacks the signal required for its activity. Our observation that this activating signal can be provided by overexpression of the enzyme may indicate that expression of the lipases is rate limiting for their in vivo activity. Thus, lipase activity in yeast may be controlled primarily at the transcriptional level. This would be in contrast to the situation of mammalian cells in which an interplay between components that localize to the lipid droplets, such as perilipin and the lipase, in this case the hormone-sensitive lipase, is important in coordinating substrate access. This interplay is regulated by protein kinase A to increase the rate of lipolysis 30- to 100-fold (21).

Even though yeast lipid particles lack any obvious perilipin orthologue, it is interesting to note that both Yeh1 and Tgl1, but not Yeh2, contain potential cyclic AMP-dependent protein kinase A phosphorylation sites. Additional studies will now be required to determine how the activity and substrate access of the yeast lipases are regulated.

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