YEH2/YLR020c Encodes a Novel Steryl Ester Hydrolase of the Yeast Saccharomyces cerevisiae

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Previous work from our laboratory (Zinser, E., Paltau, F., and Daum, G. (1993) J. Bacteriol. 175, 2853–2858) demonstrated steryl ester hydrolase activity in the plasma membrane of the yeast Saccharomyces cerevisiae. Here, we show that the gene product of YEH2/YLR020c, which is homologous to several known mammalian steryl ester hydrolases, is the enzyme catalyzing this reaction. Deletion of yeast YEH2 led to complete loss of plasma membrane steryl ester hydrolase activity whereas overexpression of the gene resulted in a significant elevation of the activity. Purification of enzymatically active Yeh2p close to homogeneity unambiguously identified this protein as a steryl ester hydrolase and thus as the first enzyme of this kind characterized in S. cerevisiae. In addition to evidence obtained in vitro experiments in vivo contributed to the characterization of this novel enzyme. Sterol analysis of yeh2A unveiled a slightly elevated level of zymosterol suggesting that the esterified form of this sterol precursor is a preferred substrate of Yeh2p. However, in strains bearing hybrid proteins with strongly enhanced Yeh2p activity decreased levels of all steryl esters were observed. Thus, it appears that Yeh2p activity is not restricted to distinct steryl esters but rather has broad substrate specificity. The fact that in a yeh2A deletion strain bulk steryl ester mobilization occurred at a similar rate as in wild type suggested that Yeh2p is not the only steryl ester hydrolase but that other enzymes with overlapping function exist in the yeast.

Most eukaryotic cells contain and synthesize sterols, which are essential lipid components of membranes. Besides their role in maintaining membrane permeability and fluidity, effects of sterols on aerobic metabolism (1, 2), completion of the cell cycle (3) as well as on sterol uptake (4) and sterol transport (5) have been described. Considering the different functions of sterols it is obvious that sterol homeostasis, including sterol biosynthesis, uptake, transport, storage, utilization, and efflux, has to be a strictly regulated process. Specific sterols present in different eukaryotic cells vary, ergosterol being the major sterol in the yeast Saccharomyces cerevisiae.

Besides other regulatory mechanisms esterification of sterols and hydrolysis of steryl esters (STE)1 play an important role in cellular sterol homeostasis. These processes do not only allow cells to store chemical energy, which can be used in times of deprivation, but also provide an additional means to balance the concentrations of free sterols and fatty acids, which are essential and critical for cell structure and function. In mammalian cells esterification of sterols is catalyzed either by lecithin:cholesterol acyltransferase (reviewed in Refs. 6–8) or by the two acyl-CoA:cholesteryl acyltransferases ACAT1 and ACAT2 (reviewed in Ref. 9). Cholesteryl ester hydrolysis occurs by a number of mammalian enzymes many of which have already been identified, among them carboxyl ester lipase (CEL), lysosomal acid lipase (LAL) and hormone-sensitive lipase (HSL) (reviewed in Ref. 10–14). Great efforts have been made to clarify the relationship between the different proteins, especially with respect to their tissue distribution and their similarities in structure and function. CEL, a protein, which is synthesized primarily in pancreatic acinar cells and lactating mammary glands of higher mammals, is involved in dietary fat and cholesterol absorption. At the same time, a possible role of CEL in plasma lipoprotein metabolism and atherosclerosis has been discussed. LAL is a key enzyme in the intracellular degradation of triacylglycerols (TAG) and cholesteryl esters derived from plasma lipoproteins. Lack of this acidic lipase activity in humans causes two distinct phenotypes, Wolman disease and cholesteryl ester storage disease. Until recently, hormone-sensitive lipase (HSL) has been regarded as an adipose tissue-specific enzyme entirely focused on the hormone-stimulated lipolysis of TAG in this tissue. Ongoing research, however, revealed that HSL plays a number of additional roles in lipid metabolism, including that of a neutral cholesteryl ester hydrolase. Finally, it has to be mentioned that similar to HSL, also CEL and LAL are not restricted to cholesteryl ester utilization but rather show broad substrate specificity.

In the yeast S. cerevisiae, sterols that are not immediately needed as membrane components can be esterified in the endoplasmic reticulum by the STE synthases Are1p and Are2p (15–18). Together with TAG, STE are stored in lipid particles and form the hydrophobic core of this compartment (reviewed in Ref. 19). Little is known, however, about pathways and mechanisms of STE hydrolysis in yeast. Whereas Taketani et al. (20) detected the highest specific activity of STE hydrolase of S. cerevisiae in mitochondria, Zinser et al. (17) reported

1 The abbreviations used are: STE, steryl esters; CEL, carboxyl ester lipase; LAL, lysosomal acid lipase; HSL, hormone-sensitive lipase; TAG, triacylglycerols; GFP, green fluorescent protein; TEV, tobacco etch virus; PAM, plasma membrane-associated membrane; YEH, yeast steryl ester hydrolase; ORF, open reading frame; GC, gas chromatography; MS, mass spectrometry; MD, mean deviation.
localization of the highest specific activity of this enzyme in the plasma membrane and secretory vesicles. It has to be considered, however, that similar to mammalian STE hydrolases enzymes with overlapping function may also occur in the yeast differing from each other with respect to their subcellular location and/or their substrate specificity.

To identify protein(s) involved in yeast STE hydrolysis we decided to screen the Saccharomyces cerevisiae genome for ORF-containing sequences which are homologous to known mammalian STE hydrolases from various organisms. Plasma membrane preparations of yeast strains lacking candidate gene products were subjected to activity assays in vitro with the aim to discover mutants with reduced or without STE hydrolase activity in this subcellular fraction. This approach identified the gene product of YEH2/YLR020c as a yeast plasma membrane located STE hydrolase through experiments in vivo and in vitro using a yeast Δ deletion mutant and various strains harboring modified Yeh2p with high STE hydrolase activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Culture Conditions, and Isolation of Subcellular Fractions**—The wild-type yeast strain S. cerevisiae BY4742 (MATa, his3Δ1, leu2Δ0, ura3Δ0, and the corresponding deletion ylr020cΔ::kanMX (EUROFAN collection, Frankfurt, Germany) were used throughout this study. Cells were grown in rich medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD) or 2% galactose (YPGal), or in minimal medium containing 0.67% yeast nitrogen base and 2% glucose (MMGal) or 2% galactose (MMGal) supplemented with the appropriate amino acids for 16 h (YPD), 22 h (YPGal), or 44 h (MMGal). For isolation of mitochondria yeast cells were grown on YPLac (2% lactate) (21).

Subcellular fractions were prepared by published procedures (22–24). Protein was quantified by the method of Lowry et al. (25) using bovine serum albumin as a standard.

**Phenotypic Analysis**—The following drugs and reagents were added to MMGal containing 2% agar immediately before pouring into Petri dishes: 0.1–4 μg/ml cycloheximide (stock in ethanol), 5–20 μg/ml fluconazole (Diflucan IV, Pfizer), 0.25–6 μg/ml nystatin (stock in N,N-dimethylformamide), 0.25–1.5 M sorbitol, 0.25–2% Brij58, 1–8% ethanal, and 0.4–4.9 mM hydrogen peroxide. Solid media were incubated for 3–4 days at 30 °C. Temperature sensitivity was tested by cultivating yeast strains on YPD, MMGal, or YPGal agar plates at 37 °C.

**Data Base Search and Computational Analysis**—Sequence alignments were performed with polypeptide sequences of known STE hydrolases from higher eukaryotic organisms like mouse, rat, and human using a BLAST search. A cut-off value of E = 0.01 to 0.05 was defined (26). Molecular data about proteins were obtained from the Inycte Proteome BioKnowledge Library, the Saccharomyces cerevisiae Gene DB, the Protein families database of alignments and HMMs (PFAM), and Swiss Prot. Transmembrane regions were predicted either by TMHMM (v2.0) (PFAM) (27, 28) or using applications available at EMBOSS (SGD).

**Lipid Analysis**—Lipids from yeast cells grown on YPD or MMGal were extracted as described by Folch et al. (29). For the quantification of neutral lipids extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (Linomat IV, CAMAG, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by using the solvent system light petroleum/diethyl ether/acidic acid (25:1:4; v/v) for the first third of the distance. Then, plates were dried briefly and further developed to the top of the plate using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of erosteryl and erester esters was carried out by densitometric scanning at 275 nm using a Shimadzu dual-wavelength chromatocanner CS-930 with ergosterol as a standard. TAG was visualized by postchromatographic staining with a chromatogram immersion device (CAMAG). Plates were dipped for 6 s into a developing reagent consisting of 0.63 g of MnCl2·4H2O, 60 ml of H2O, 60 ml of methanol, and 4 ml of concentrated sulfuric acid, briefly dried, and heated at 100 °C for 30 min. Quantification of TAG was carried out by densitometric scanning at 400 nm with triolein as a standard.

For gas liquid chromatography/mass spectrometry (GC/MS) of sterols (18) either whole cells corresponding to 20 ml of cells with an OD600 of 1 or 500 μg of homogenate protein were incubated for 2 h at 90 °C together with 0.6 ml of methanol, 0.4 ml of 0.5% pyrogallol dissolved in methanol, 0.4 ml of 60% aqueous KOH, and 10 μg of cholesterol dissolved in ethanol as an internal standard. Lipids were extracted three times with n-heptane and combined extracts were taken to dryness under a stream of nitrogen. Then, lipids were dissolved in 10 μl of pyridine and after adding 10 μl of O-(trimethylsilyl)trifluoroacetamide (Sigma) samples were diluted with ethyl acetate to an appropriate concentration. GC/MS analysis of silylated sterol adducts was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA), equipped with an HP5972 mass selective detector, and HP 5-MS column (cross-linked 5% phenyl methyl silicone; dimension 30 m × 0.25 mm × 0.25 μm film thickness. 0.25 ml of 1 μl were injected in the splitless mode at 270 °C injection temperature with helium as carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100 °C, 10 °C/min to 250 °C, and 3 °C/min to 310 °C. Mass spectra were acquired in scan mode (scan range 200–550 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

**Lipid Analysis in Vitro and Mobilization of Sterol Ester in Vivo**—STE hydrolase activity was determined as described by Taketani et al. (20) and Zinser et al. (17). For standard substrate preparation 165 μl of cholesterol oleate (1 mg/ml in CHCl3/Methanol, 2:1, v/v), 50 μl of cholesterol [1,14C]oleate (100 μCi/ml) and 150 μl of Triton X-100 (100 mg/ml in methanol) were mixed and taken to dryness. The remnant was suspended in 2.5 ml of Tris-HCl buffer, pH 7.4. STE hydrolase activity was estimated in a total volume of 0.5 ml containing 0.25 ml of the aqueous suspension of radiolabeled cholesterol oleate (see above) and 0.3–1 μg of protein. The reaction was linear with time for at least 10 min. Assays were stopped by adding 4 ml of chloroform/methanol (2:1, v/v), and lipids were extracted (29) and neutral lipids were separated by thin-layer chromatography using the solvent system light petroleum/diethyl ether/acidic acid (35:15:1; per vol.). Bands corresponding to free fatty acids and STEn were scraped off the thin-layer plates, and radioactivity was determined by liquid scintillation counting.

For the analysis of enzyme kinetics, rates of metabolic conversion were determined at different substrate concentrations. Variable amounts of cholesterol oleate (1 mg/ml in CHCl3/Methanol, 2:1, v/v) together with 5 μl of cholesterol [1,14C]oleate (100 μCi/ml) and 45 μl of Triton X-100 (100 mg/ml in methanol) were used as substrate in a final volume of 0.25 ml of Tris-HCl buffer, pH 7.4.

Substrate specificity of Yeh2p was tested by using either 15 μg of TAG labeled with [14C]palmitic acid, or 125 nmol phospholipids labeled with [14C]oleate as substrate under the same assay conditions as described above. Wild-type levels of fatty acids released from these substrates were compared with a strain overexpressing Yeh2p. For the biosynthesis of [14C]oleate 100 μl of synthetic medium containing either 20 μCi of [14C]palmitic acid or [14C]oleate (0.4 μmol, respectively), were inoculated with 200 μl of a wild-type preculture grown for 48 h. After 24 h of cultivation, cells were harvested, and lipids were extracted as described above. Lipids were separated by thin layer chromatography using light petroleum/diethyl ether/acidic acid (35:15:1, per volume) as a solvent. Bands of TAG or phospholipids were scraped off the thin-layer plates, and radioactivity was determined by the method of Broekhuysen (31).

Mobilization of STE in vivo was tested as described by Leber et al. (32). In brief, yeast cells were pregrown in YPD medium for 16 h and transferred to sterol-free medium (MMGlu). Terbinafine dissolved in ethanol was added at a final concentration of 30 μg/ml. The optical density was measured with a Hitachi U-1100 UV/VIS Spectrophotometer at a wavelength of 600 nm, and aliquots of the cultures were withdrawn at different time points. Cells were harvested by centrifugation, washed once with H2O, and prepared for lipid analysis.

**Cloning and Tugging of YEH2**—YEH2 was cloned into the vector pUC19 for homologous recombination. For this fragment of the ORF expressed 3′ and 5′ by 60 bp homologous to pGP316 together with the linearized vector were transformed (33) into the wild-type strain S. cerevisiae BY4742. Positive transformants were selected by uracil auxotrophy.

YEH2 was tagged in the genome of wild-type BY4742 by transformation (33) with PCR products from the template plasmids pFA6a-GFP/S65T-kanMX6 (C-terminal GFP tag, pFA6a-kanMX5-PGAL1

2 The nomenclature of the ORF YLR020c as YEH2 was chosen in agreement with the SGD data base (www.yeastgenome.org/).
Yeast Steryl Ester Hydrolase

TABLE I
Primers used for different YEH2 constructs

| Sequence 5′−3′ |
|----------------|
| pGP316+YEH2 (fw) | TACCTCTTAACATTCCTACGAGGAAAAACCCCCGAGGTTCTGCTGATGTGTTGTTGGATOT |
| pGP316+YEH2 (rev) | GATCGGCTGTGACAGTCAGGATCAGAGGAAAATTTGAGGTTTCCTAATGGCAAGGGGAGCAGGGGCGGGTGC |
| C-terminal GFP tag (fw) | AAGATGTTGATCAACTGCTTACCTCATCATCAACCCCTTATTTACATGATGATGATGATGCATTTTGAGATCCGGGTTTT |
| C-terminal GFP tag (rev) | TTATGGCATATTATTTTACAAAGAAACCACAAAGAAAAAACTTTTACCGAATTCGAGCTCGTTTAAAC |
| N-terminal His tag + gal-prom (fw) | AGAATTCGACACCTGACCCCACTCATCATCAACCCCTTATTTACATGATGATGATGATGCATTTTGAGATCCGGGTTTT |
| N-terminal His tag + gal-prom (rev) | see N-terminal tag + gal-prom (fw) |
| 6× Histidine tag (fw) | TTATGGCATATTATTTTACAAAGAAACCACAAAGAAAAAACTTTTACCGAATTCGAGCTCGTTTAAAC |
| 6× Histidine tag (rev) | see 6× Histidine tag (fw) |

(insertion of a gal-promoter), pFA6α-His3MX6-PGA1L (insertion of a gal-promoter) (34) and YCPlac23-ZZ (35) (C-terminal zr-tag). The N-terminal histidine tag was introduced by additional primer bases. For all primers used see Table I. The different constructs and the respective recombinant strains used in this study are referred to as Yeh2p-GFP/ YEH2-GFP, His-Yeh2p/His-YEH2 and Yeh2p-protA/YEH2-protA. The correctness of insertions was tested either by resistance of strains to geneticin or by marker-degenerate growth. Additionally, identity of all strains was confirmed by colony PCR.

Purification of Yeh2p—For solubilization of the protein A-tagged Yeh2p, plasma membrane preparations of the strain YEH2-protA were incubated with 5 mM sucrose monolaurate and 10% glycerol at 4 °C for 30 min. After centrifugation at 13,000 × g for 15 min at 4 °C, the supernatant was saved and the solubilization step was repeated twice with the resuspended pellets. To remove low molecular weight components the combined supernatants were subjected to ultrafiltration (Microcon YM-50; Millipore). The retentate was diluted with TST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20) and incubated with an IgG Sepharose 6 Fast Flow suspension (Amersham Biosciences), which had been pre-equilibrated according to the manufacturer’s instructions, in a 1:ml column (Mobicoll; MoBiTec) for 2 h at 4 °C. Then, beads were washed with 1–2 bed volume(s) of TST and three times with 1–2 bed volume(s) of AcTEV (Tobacco Etch Virus) buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA). Fusion protein cleavage was performed in AcTEV buffer in the presence of AcTEV™ protease (Invitrogen) for 1 h at 30 °C as suggested by the manufacturer. The major portion of the eluate was used for enzyme analysis, and a small aliquot was precipitated with 50% trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis (36).

Fluorescence Microscopy—For fluorescence microscopic analysis aliquots of strains YEH2-GFP grown in YPGal or His-YEH2 grown in MMGal were concentrated by centrifugation. GFP fusion proteins were visualized on a Zeiss Axiosvert 35 microscope using a 100-fold oil immersion objective at a detection range between 450 and 490 nm. Lipid particles were stained with Nile Red (Sigma) by standard procedures and visualized at a range from 510 to 560 nm. Images were taken with a CCD camera.

Electron Microscopy—Yeast cells grown on MMGal were sedimented by centrifugation, rinsed in 0.1 M cacodylate buffer (pH 7.4) and fixed with 3% glutaraldehyde and 0.1 M cacodylate buffer (pH 7.4) containing 5 mM CaCl₂ and 5 mM MgCl₂, for 3 h at room temperature and 3 h on ice. Then, cells were washed twice with 0.1 M cacodylate buffer (pH 7.4) and postfixed in 4% KMnO₄, prepared in H₂O distilled for 90 min. After rinsing the pellet four times, cells were treated with 0.5% sodium metaperiodate for 15 min at room temperature, washed once more with H₂O distilled, and dispersed in a 2% agarose solution (1:1). The solidified agar containing the cells was cut into small cubes of about 1 mm³ and placed for en bloc staining into 2% uranyl acetate in H₂O distilled overnight on ice. Blocks were washed with H₂O distilled and dehydrated through a graded series of acetone, 10 min each, followed by three washes with 100% acetone for 15 min each, Samples were incubated in 2:1, 1:1, and 1:2 mixtures of acetone/Spurr resin for 2 h, respectively. The last incubation step was performed overnight to allow slow evaporation of acetone. After removal of the Spurr resin, agar cubes were incubated in fresh 100% Spurr resin for another 2 h. Then, samples were placed into gelatin capsules containing fresh Spurr resin (100%) and capsules were placed in a polymerization oven at 70 °C for 48 h. Using a diamond knife (Diatome) 75-nm ultrathin sections were cut on a Leica UltracutE ultramicrotome and collected onto 75 mesh copper palladium grids with a support film of pilofoam. Sections were stained in 1% uranyl acetate in H₂O distilled for 20 min, washed and stained once more in lead citrate (37). Images were produced on a Tecnai G² 12 (FEI Company) equipped with a CCD camera (Gatan Biiscan) at 100 kV.

RESULTS

In search of STE hydrolase(s) of the yeast S. cerevisiae bioinformatic analysis identified, among several other candidate genes, the ORF YLR020c/YEH2 as a gene potentially encoding such an enzyme. Yeh2p has not been characterized by function before. This polypeptide shows similarity to human gastric lipase and lysosomal acid lipase A as well as to S. cerevisiae Tg1lp and contains an α/β hydrolase-associated lipase region in amino acid positions 146–212 (Fig. 1). Depending on the data base used for the in silico analysis Yeh2p is predicted to harbor one or two transmembrane spanning region(s) at amino acid positions 12–31 (or 11–35) and 361–389, respectively. The assumption that Yeh2p is a membrane protein was confirmed by fluorescence microscopic inspection showing that a Yeh2p-GFP fusion protein was localized to the yeast plasma membrane (Fig. 2). Moreover, enzymatic analysis with isolated subcellular fractions demonstrated highest specific STE hydrolase activity in purified plasma membrane (Table II) thus confirming previous results from our laboratory (17). In summary, bioinformatic, cell biological, and biochemical evidence strongly suggested that Yeh2p is a plasma membrane-located yeast STE hydrolase.

Yeh2p Catalyzes Steryl Ester Hydrolase Activity in Vitro—To test STE hydrolase activity of Yeh2p in vitro we used purified plasma membrane fractions from various strains grown either on full medium (YPD or YPGal) or on minimal medium (∼uraMMG). In wild type, significant enzymatic activity was detected, whereas STE hydrolase activity was completely absent from plasma membrane preparations isolated from a yeh2Δ deletion mutant (Table III). Overexpression of YEH2 using plasmid pGP316 as a transformation vehicle led to significant elevation of the enzymatic activity. Even higher STE hydrolase activity was measured in strains harboring His-Yeh2p and Yeh2p-protA hybrid proteins. In contrast to Yeh2p-protA, His-Yeh2p was not significantly overexpressed (data not shown). The high enzymatic activity of the latter polypeptide, however, was not surprising. Structural modifications caused by introduction of the N-terminal tag may by chance “positively” modify the active center of Yeh2p or its structural environment thereby enhancing the enzymatic activity as has been shown in many other cases before.

Data presented so far strongly supported the identification of Yeh2p as a STE hydrolase, but did not fully prove it. A role for this polypeptide as an activator or regulator of the hydrolytic reaction could not be ruled out completely by the experiments described above. To address this question we made use of the Yeh2p-protA fusion protein, which was significantly overexpressed in the respective recombinant strain and accumulated at a considerable amount in the plasma membrane (Fig. 3A). Yeh2p-protA was isolated by affinity chromatography, cleaved
as described under “Experimental Procedures” and subjected to protein and enzymatic analysis. As can be seen from Fig. 3B this purification protocol allowed isolation of Yeh2p close to homogeneity. Most importantly, the isolated polypeptide exhibited a STE hydrolase activity of 42.2 nmol oleic acid released from cholesteryl oleate per min and mg of protein. This result confirmed that Yeh2p is the enzymatically active enzyme, and YEH2 is the structural gene for a yeast STE hydrolase.

Substrate Specificity and Enzymatic Properties of Yeh2p—Substrate specificity in vitro was tested with regard to potential TAG lipase and/or phospholipase activity of Yeh2p. For this purpose hydrolysis of TAG and phospholipids was measured using purified plasma membrane fractions from wild type or YEH2-protA, respectively, as enzyme sources. Whereas the amount of fatty acids released from TAG was below the detection limit for both samples, YEH2-protA revealed a slightly higher phospholipase activity (0.06 nmol of fatty acids released/min) compared with wild-type (0.04 nmol fatty acids released/min). However, this increase of activity was marginal compared with that of STE hydrolase (see Table III). Thus, we assume that besides its major function as STE hydrolase Yeh2p exhibits minor phospholipase activity.

To characterize enzymatic properties of Yeh2p in more detail we carried out analysis of enzyme kinetics. As can be seen from Fig. 4, our measurements yielded apparent values for $V_{\text{max}} \sim 2.3$ nmol/ml/min and $K_m \sim 121$ nmol/ml. One of the major difficulties in studying enzymes acting in or on membranous structures is that reactions occur in two-dimensional space rather than in traditionally studied three-dimensional solutions (for a review see Ref. 38). Taking this limitation into account, data obtained by standard kinetic assays have to be interpreted with caution.

Function of Yeh2p in Vivo—To obtain information about the cell biological and biochemical role of Yeh2p in vivo we first analyzed the basic growth phenotype of the yeh2 strain. This analysis revealed that the mutant grew on various carbon sources such as dextrose, galactose, glycerol, and lactate like wild-type and was not temperature-sensitive. Second, we
Plasma membrane from wild-type BY4742. 

**ProtA bound to IgG Sepharose 6 Fast Flow with AcTEV™ protease**

crude plasma membrane isolated from 30,000 g

Mitochondria Porin 0.06

Microsomes 40,000 g

100,000 g

Lipid particles

**Ayr1p** Not detectable

Cytosol **GAPDH**

**TABLE II**

**Subcellular distribution of steryl ester hydrolase**

| Subcellular fraction | Marker protein | Specific activity nmol oleic acid released/mg protein/min |
|----------------------|----------------|--------------------------------------------------------|
| Homogenate           | PM-ATPase<sup>a</sup> | 0.002                                                   |
| Plasma membrane      | PM-ATPase<sup>b</sup> | 0.19                                                    |
| (fraction I)         | PM-ATPase<sup>c</sup> | 0.26                                                    |
| Mitochondria         | Sec61p          | 0.08                                                    |
| Microsomes           | 30,000 g        | 0.04                                                    |
| Microsomes           | 40,000 g        | 0.04                                                    |
| Microsomes           | 100,000 g       | 0.02                                                    |
| Lipid particles      | Ayr1p           | Not detectable                                         |
|                      | GAPDH<sup>c</sup> | 0.03                                                    |

<sup>a</sup> Plasma membrane fraction I was obtained by employing one step of gradient purification; plasma membrane fraction II was obtained by employing two steps of gradient purification.

<sup>b</sup> PM-ATPase: plasma membrane ATPase.

<sup>c</sup> GAPDH, glyceraldehyde phosphate dehydrogenase.

**TABLE III**

**In vitro STE hydrolase activity in yeast plasma membrane preparations**

| Strain            | Medium            | Specific activity nmol oleic acid/ mg protein/min |
|-------------------|-------------------|-------------------------------------------------|
| BY4742 (wild type)| YPD               | 0.2                                             |
| BY4742 (wild type)| YPDGal            | 0.4                                             |
| BY4742 (wild type)| YPGal-uraMMGal    | Not detectable                                 |
| + pGP316 (empty  | plasmid)          |                                                  |
| yeh2Δ             | YPD               | Not detectable                                 |
| BY4742 (wild type)| -uraMMGal         | 2.1                                             |
| His-YEH2          | YPGal             | 12.4                                            |
| YEH2-protA        | YPGal             | 39.8                                            |

**FIG. 3. Overexpression of Yeh2p-protA and isolation of Yeh2p.**

A, Yeh2p-protA was significantly overexpressed and localized to the plasma membrane fraction. The band seen on the SDS-polyacrylamide gel marked by the asterisk (*) has the expected molecular mass of 75 kDa of the fusion protein as deduced from molecular masses of Yeh2p<sub>asterisk</sub> (62 kDa) and zz-tag (13 kDa).

B, Yeh2p-protA was significantly overexpressed and localized to the plasma membrane fraction. The band seen on the SDS-polyacrylamide gel marked by the asterisk (*) has the expected molecular mass of 75 kDa of the fusion protein as deduced from molecular masses of Yeh2p<sub>asterisk</sub> (62 kDa) and zz-tag (13 kDa).

examined the lipid profile of the yeh2Δ strain. The mutant lipid composition was not different from wild-type with respect to the amounts of TAG, ergosterol esters, and free ergosterol, and the fatty acid pattern (data not shown). Closer analysis of the sterol composition, however, revealed a 15% elevated level of zymosterol in the mutant compared with wild type (Table IV).

Since zymosterol as other sterol precursors is known to occur obviously not the major one. Thus, we propose that additional enzymes involved in STE hydrolysis must exist in the yeast.

**TABLE IV**

**Sterol analysis of yeh2Δ compared to the wild-type BY4742**

Cholesterol was used as an internal standard for GCMS estimation of sterols as described under "Experimental Procedures."

| Sterol             | Relative amount BY4742 | yeh2Δ | Ratio of relative amounts of sterols in yeh2Δ to BY4742 |
|--------------------|------------------------|-------|--------------------------------------------------------|
| Zymosterol         | 0.421                  | 0.484 | 115                                                    |
| Ergosterol         | 1.948                  | 1.967 | 101                                                    |
| 4-Methylzimosterol | 0.282                  | 0.245 | 87                                                     |
| Fecosterol         | 0.977                  | 0.879 | 90                                                     |
| Episterol          | 0.307                  | 0.276 | 90                                                     |
| Lanosterol         | 0.029                  | 0.029 | 97                                                     |

in yeh2Δ was accompanied by a 10% decrease of fecosterol and episterol, the next intermediates following zymosterol in the ergosterol biosynthetic pathway. At the same time a reduced level of 4-methylzimosterol was observed in the deletion mutant (see Table IV).

Results described above tempted us to speculate that Yeh2p may be a STE hydrolase specific for esterified sterol precursors. To test this hypothesis we performed experiments in <i>vivo</i> aimed at the documentation of STE, in particular zymosterol ester, mobilization. For this purpose overnight cultures of yeh2Δ and wild type were shifted to a sterol-free medium and then treated with the ergosterol biosynthesis inhibitor terbinafine. Under these conditions, depletion of cellular sterols leads to mobilization and hydrolysis of STE (32). These experiments clearly demonstrated that ergosterol esters and sterol precursor esters including zymosterol esters were hydrolyzed in yeh2Δ at the same rate as in wild type (data not shown). Although this appeared to be a negative result at the first sight, it provided important information. We concluded from these data that Yeh2p is not the only STE hydrolase in yeast and obviously not the major one. Thus, we propose that additional enzymes involved in STE hydrolysis must exist in the yeast.
Data are mean values from three independent experiments with an MD of ±10%. The relative amounts of total sterols (free and esterified) from the wild-type BY4742 (white bars) and the mutant His-YEH2 (black bars). The amounts of the different sterols found in the wild type were arbitrarily set at 100%. Data are mean values from three independent experiments with an MD of ±5%. Zym, zymosterol; Erg, ergosterol; Fec, fecosterol; Lan, lanosterol.

the sterol composition. In total cell extracts from the His-YEH2 strain the amount of ergosterol esters was reduced to ~50% of wild-type (Fig. 5A), whereas the level of free ergosterol was almost unchanged. Thus, the reduced amount of total ergosterol in His-YEH2 (Fig. 5B) was completely attributable to the decrease of the esterified form. Besides ergosterol, total amounts of sterol precursors were even more significantly decreased in His-YEH2. The most dramatic alteration was observed with zymosterol, which dropped practically to zero in the mutant. Taking into account that sterol intermediates are almost exclusively present in the form of STE, our results indicate that Yeh2p hydrolyzes zymosterol esters with some preference, but at the same time also uses other sterol precursor esters and ergosterol esters as appropriate substrates.

Cell Structure of Strains with Enhanced Steryl Ester Hydrolase Activity—To test possible effects of imbalanced sterol/STE levels on the cellular structure of the yeast we performed a microscopic inspection of the respective strains with special emphasis on the two compartments which are mainly involved in sterol homeostasis, lipid particles, and plasma membrane. Recent experiments from our laboratory (39) had shown the highly flexible formation of lipid particles even in the absence of STE or TAG synthesis, respectively. Therefore, it was not surprising that number and size of lipid particles in His-YEH2 were roughly the same as in wild type when visualized by fluorescence microscopy using the neutral lipid specific fluorescence dye Nile Red (data not shown). In contrast, significant changes of the plasma membrane structure were observed when His-YEH2 was subjected to electron microscopic inspection (Fig. 6A). The membranous structures clearly identified as plasma membrane and peripheral endoplasmic reticulum (40) in wild type appeared to fuse in His-YEH2 resulting in punctuate areas. In contrast to the continuous membrane system seen in wild type the plasma membrane of His-YEH2 looked fuzzy and rigid. This structural feature may also be the reason why several attempts failed to isolate plasma membrane from His-YEH2 grown on MMGalen by standard procedures.

Changes in the structure of the yeast plasma membrane often result in altered sensitivity of cells to drugs or other stress-inducing agents present in the environment. For this reason, we performed growth tests in the presence of sorbitol (osmotic stress), hydrogen peroxide (oxidative stress), Brij 58 (detergent), cycloheximide (inhibitor of protein biosynthesis), ethanol (non-fermentable carbon source), fluconazol (inhibitor of ergosterol biosynthesis), nystatin (polyene antibiotic) and at 37 °C (temperature stress) with His-YEH2, YEH2-protA (high Yeh2p activity), and the deletion mutant yeh2Δ (lack of Yeh2p activity). These experiments demonstrated that both His-YEH2 and YEH2-protA were sensitive to nystatin, whereas yeh2Δ was less susceptible to this drug than wild-type cells (Fig. 6B). Furthermore, all three strains, yeh2Δ, His-YEH2 and YEH2-protA, showed slightly elevated resistance to hydrogen peroxide. All other reagents or conditions tested did not affect the growth behavior of the mutants compared with wild type (data not shown). The polyene component nystatin, similar to the antifungal drug amphotericin B, preferentially binds to ergosterol in membranes (41). Thus, changes in nystatin sensitivity are indicative of a disturbed structure of the plasma membrane in particular due to alterations of sterol incorporation into this compartment. This view is in line with our observation that in strains with enhanced STE hydrolase activity the plasma membrane structure is altered as shown by electron microscopy (see above).

DISCUSSION

The study presented here describes for the first time identification and characterization of the plasma membrane STE hydrolase Yeh2p from the yeast S. cerevisiae. This discovery is based on the following evidence: First, Yeh2p harbors an α/β hydrolase fold that is common to several hydrolytic enzymes from various species (42). Second, the bioinformatically predicted transmembrane region(s) present in Yeh2p, the measurement of the enzymatic activity in the plasma membrane and the fluorescence microscopic localization of a Yeh2p-GFP hy-
brid clearly attributed this enzyme to the plasma membrane. Third and most importantly, plasma membrane preparations from the yeh2Δ deletion mutant lost the ability to hydrolyze STE in vitro, whereas the isolated polypeptide exhibited STE hydrolase activity. Finally, experiments in vivo especially with strains harboring enhanced Yeh2p activity demonstrated that this enzyme is able to hydrolyze STE with a slight preference for zymosteroyl esters.

Some phenotypic changes in strains with high activity of Yeh2p grown on minimal medium provided supplementary evidence for the involvement of this protein in yeast lipid metabolism. These strains not only exhibited altered susceptibility to nystatin but also suffered from severe plasma membrane disturbance (Fig. 6). Therefore, it appears that His-YEH2 plasma membrane fractions eluded isolation by conventional procedures the respective analysis could not be properly performed. Excessive STE hydrolysis in the presence of genetically modified Yeh2p leading to local accumulation of sterols or sterol intermediates, or even the presence of the Yeh2p hybrid itself may be taken into account as possible reasons for the observed effects.

Our results also raised the question as to the physiological relevance of a STE hydrolase localized to the plasma membrane. It came as a surprise that such an enzyme was not associated with lipid particles, the site of STE storage and the obvious starting point for STE mobilization. A related process, the mobilization of TAG from lipid particles, was recently shown to be catalyzed by the lipid particle located Tgl3p, the major TAG lipase of S. cerevisiae (43). On the other hand, the mammalian hormone sensitive lipase HSL, which catalyzes hydrolysis of TAG, diacylglycerols, monoacylglycerols, lipoidal, and retinyl esters and also cholesteryl esters (44–46), is a cytosolic protein under basal conditions. Only upon lipolytic stimulation of adipocytes a rapid and dramatic redistribution of HSL occurs which result in the movement of the protein to the surface of lipid droplets (47–49). Similar regulatory processes may be relevant for the yeast. Thus, STE hydrolyzing enzymes need not necessarily be standard components of the lipid storage particles. Since the plasma membrane is the yeast organelle with the highest concentration of free sterols, enzymes setting sterols free from STE may also be considered to be components of this fraction. Whereas in adipocyte lipolysis the hydrolysis approaches the storage compartment, in the yeast the STE-containing lipid particles may migrate to the site of the hydrolytic enzyme. This hypothesis is supported by recent work from our laboratory proposing a role of lipid particles in the transport of ergosterol to the yeast plasma membrane (39). This study showed that the amount of free ergosterol in the plasma membrane of a strain deleted of all neutral lipid-synthesizing genes, which is unable to form lipid particles, was only 60% compared with wild type. Thus, lipid particles may migrate to the plasma membrane upon requirement, e.g. upon shortage of sterols, and get access to a plasma membrane associated STE hydrolase.

Another point of discussion raised by our findings is the physiological relevance of a plasma membrane located STE hydrolase setting free not only ergosterol but also sterol precursors. The most prominent sterol in the yeast plasma membrane is ergosterol, although zymosterol can be found in minor quantity in this membrane as well (17). Therefore, it appears unlikely that a plasma membrane associated STE hydrolase forms a huge amount of zymosterol or other sterol precursors to be incorporated into the plasma membrane under normal conditions. Consequently, there is a need to convert these sterol intermediates to the end product of the biosynthetic pathway, ergosterol. These steps cannot occur in the plasma membrane itself, but may take place in PAM (plasma membrane associated membrane). PAM is a subfraction of the endoplasmic reticulum in close proximity to the plasma membrane, which was found to be highly enriched in a subset of sterol-synthesizing enzymes (40). Besides other enzymes, the sterol-C24-methyltransferase Erg6p, which converts zymosterol to fecosterol is present in PAM at even higher specific activity than in bulk endoplasmic reticulum. Since PAM is linked to the bulk endoplasmic reticulum an excess of zymosterol set free in the plasma membrane may first be incorporated into PAM, probably by membrane contact, and then move to any other part of the endoplasmic reticulum where completion of ergosterol synthesis occurs. Notably, Are1p and Are2p, the two enzymes catalyzing the synthesis of STE, are not enriched in PAM (40), which avoids a futile cycle in this compartment.

STE mobilization assays performed with wild-type cells and yeh2Δ disclosed the existence of a complex STE hydrolytic machinery in yeast, which does not only consist of Yeh2p. Under sterol depletion, STE of lipid particles were perfectly hydrolyzed even in the absence of Yeh2p indicating the existence of additional and even major members of this hydrolase family. The presence of STE hydrolyase isoenzymes in yeast was also suggested by the fact, that levels of STE with the exception of zymosterol esters were not increased in yeh2Δ. It has to be taken into account, however, that enzymes with overlapping enzymatic activity may be up-regulated when one enzyme of the STE hydrolytic machinery is deleted. Most recently, indeed, two additional yeast STE hydrolases were identified in our laboratory and by others (50). Characterization of the STE hydrolase family will be paramount in the future and lead us to a better understanding of regulatory mechanisms governing sterol homeostasis in the yeast.

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