Membrane Topology and Essential Amino Acid Residues of Phs1, a 3-Hydroxyacyl-CoA Dehydratase Involved in Very Long-chain Fatty Acid Elongation*§

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Yeast Phs1 is the 3-hydroxyacyl-CoA dehydratase that catalyzes the third reaction of the four-step cycle in the elongation of very long-chain fatty acids (VLCFAs). In yeast, the hydrophobic backbone of sphingolipids, ceramide, consists of a long-chain base and an amide-linked C26 VLCFA. Therefore, defects in VLCFA synthesis would be expected to greatly affect sphingolipid synthesis. In fact, in this study we found that reduced Phs1 levels result in significant impairment of the conversion of ceramide to inositol phosphorylceramide. Phs1 proteins are conserved among eukaryotes, constituting a novel protein family. Phs1 family members exhibit no sequence similarity to other dehydratase families, so their active site sequence and catalytic mechanism have been completely unknown. Here, by mutating 22 residues conserved among Phs1 family members, we identified six amino acid residues important in Phs1 function, two of which (Tyr-149 and Glu-156) are indispensable. We also examined the membrane topology of Phs1 using an N-glycosylation reporter assay. Our results suggest that Phs1 is a membrane-spanning protein that traverses the membrane six times and has an N terminus and C terminus facing the cytosol. The important amino acids are concentrated in or near two of the six proposed transmembrane regions. Thus, we also propose a catalytic mechanism for Phs1 that is not unlike mechanisms used by other hydratases active in lipid synthesis.

Sphingolipids are abundant lipid components of eukaryotic plasma membranes that have roles in a wide range of biological processes, such as proliferation, apoptosis, differentiation, cell cycle control, adhesion, and intracellular trafficking (1–3). Ceramide, the backbone of sphingolipids, is composed of a long-chain base (LCB) attached to a fatty acid (FA) via an amide bond. In mammals, FA chain length ranges from C14 to C26, except in certain tissues such as skin, testis, and sperm, which have even longer FAs (4–7). In most tissues, however, C16 (C16:0) FA is predominant, with the C24 (C24:0 and C24:1) species following. In contrast, in the yeast *Saccharomyces cerevisiae* the chain length of the FA moiety in CER is predominantly C26. FAs with chain length of 20 or longer are known as very long chain (VLCFAs), and they themselves function in numerous cellular processes, including glycosylphosphatidylinositol anchor biogenesis (8), maintenance of a functional nuclear envelope (9, 10), protein transport (11), and production of signaling molecules such as arachidonic acid (12). In fact, yeast mutants deficient in VLCFA production are inviable (13, 14), indicating that VLCFAs perform essential functions that cannot be substituted for by more common long-chain FAs (LCFAs) such as C16 and C18. Furthermore, any impairment of VLCFA production also affects the function of those sphingolipids that carry these long chains.

In yeast or mammals, the synthesis of LCFAs is carried out by a multienzyme known as soluble fatty-acid synthase (FAS) (15). FA elongation occurs by cycling through a four-step process (condensation, reduction, dehydration, and reduction), during which the FA chain is bound covalently to the acyl carrier protein (ACP) domain of FAS. Mammalian and yeast FAS (type I FAS) incorporate all catalytic activities of the cyclic reaction as discrete domains on one and two polypeptide chain(s), respectively (15, 16). In bacteria, plants, and mitochondria, however, FAS (type II) includes a dissociated system wherein each component is encoded by a separate gene (17). Mammalian and yeast LCFA can be further converted to VLCFAs by an endoplasmic reticulum (ER) membrane-bound elongase complex (18). This reaction is also carried out by cycling through a four-step process similar to that performed by FAS (Fig. 1); however, malonyl-CoA and acyl-CoA are not covalently attached to the complex but exist as separate compounds. The yeast elongase complex is composed of at least four different polypeptide chains. The 3-hydroxyacyl-CoA dehydratase Phs1, which is...
responsible for the third step, catalyzes the dehydration of the 3-hydroxyacyl-CoA (18).

Phs1 was first reported as a factor involved in sphingolipid metabolism (19). This study found that decreases in Phs1 levels were accompanied by increases in the LCBs dihydrosphingosine and 3-ketodihydrosphingosine (DHS) and phytosphingosine (PHS) and in their phosphorylated forms, the LCB phosphates (19); in fact, LCB accumulation is a general phenotype of VLCFA synthesis mutants (14, 20). Phs1 is highly conserved among eukaryotes, constitutively expressed (22). Phs1 mRNA is restricted to heart and muscle (21), whereas PTPLA and PTPLB. Expression of PTPLA and PTPLB. Expression of PTPLA mRNA is restricted to heart and muscle (21), whereas PTPLB mRNA is ubiquitously expressed (22). PTPLA has been linked to certain muscle diseases, and disruption of the dog gene results in centronuclear myopathy (23). Moreover, mutations in the human PTPLA gene were found in patients with arrhythogenic right ventricular dysplasia (21), although the relationship between the mutation and the disease is not clear.

Despite having a similar activity, Phs1 shares no sequence similarity to either the 3-hydroxyacyl-ACP dehydratase of FAS II or to the 3-hydroxyacyl-ACP dehydratase domain of FAS I. The dehydratase active sites of the FASs share a conserved His residue (17); however, no conserved His residue exists in Phs1 family members. Furthermore, Phs1 enzymes are predicted to be multispanning membrane proteins, whereas the FASs are soluble. Therefore, both the overall structures and the catalytic mechanisms of Phs1 family members and 3-hydroxyacyl-ACP dehydratase (or domain) of FASs may differ greatly. Determining the essential amino acid residues and membrane topology of Phs1 is an important step in understanding its catalytic mechanism. In this study, we changed each of 22 amino acid residues conserved in the Phs1 family to Ala. We identified six important residues, two of which were essential for Phs1 function. In addition, we examined the membrane topology of Phs1 using an N-glycosylation reporter assay, and we now propose that Phs1 is a membrane-spanning protein that traverses the membrane six times, with its N terminus and C terminus facing the cytosol.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Several S. cerevisiae strains were used. BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) (24), R1158 (BY4741, URA3::CMV-tTA) (25), and TH_3237 (R1158, pPHS1::kanMX4-TetO2; CYC7::TATA) (26) were obtained from Open Biosystems (Huntsville, AL), and SAY31 and SAY32 cells are MET15+ derivatives of R1158 and TH_3237 cells, respectively. The KanMX4 gene in the TH_3237 cell line was replaced with the LEU2 gene to produce SAY33 cells. The DEY102 line (R1158, Δpep4::MET15) was constructed by replacing the entire open reading frame of the PEP4 gene with the MET15 marker. The DEY113 (BY4741, Δpep4::MET15 Δprb1::kanMX4) cell line was constructed by replacing the entire open reading frames of the PEP4 and PRB1 genes with the MET15 and KanMX4 markers, respectively. All cells were grown in either YPD medium (1% yeast extract, 2% bactopeptone, and 2% d-glucose) or synthetic complete (SC) medium (0.67% yeast nitrogen base and 2% d-glucose) containing nutritional supplements.

Quantitative Analysis of LCB Levels—Cells from each line tested (~3.8 × 107 cells) were harvested by centrifugation and suspended in 100 μl of water. After 60 pmol of sphingosine (chain length C18) was added as an internal control, lipids were extracted from cells by successively adding and mixing 375 μl of chloroform/methanol/HCl (100:200:1, v/v), 125 μl of chloroform, and 125 μl of 1% KCl. Phases were separated by centrifugation, and the organic phase was recovered, dried, and suspended in 120 μl of ethanol by sonication and by heating for 25 min at 67 °C. The obtained lipid solution was then treated with 15 μl of OPA reagent (1 mg/ml o-phthalaldehyde and 0.2% 2-mercaptoethanol in 3% boric acid (pH 10.5)) for 1 h at room temperature. After a centrifugation at 20,000 × g for 5 min, the supernatant (10 μl) was resolved by HPLC (Agilent 1100 series; Agilent Technologies, Palo Alto, CA) on a pre-packed C18 reverse phase column (COSMOSIL 5C18-AR-II; Nakalai Tesque, Kyoto, Japan) using an isocratic eluent composition of methanol, 10 μM potassium phosphate (pH 7.2), 1 μM tetrabutylammonium dihydrogen phosphate (83:16.1, v/v), at a flow rate of 1.5 ml/min at 40 °C. Lipids modified with o-phthalaldehyde were monitored at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

In Vivo Labeling Experiments—Prior to [14C]Ser labeling, yeast cells were grown to ~1.25 × 107 cells/ml in SC medium lacking Ser and Thr, and then 1 ml of cells were labeled with 1 μCi of [14C]Ser (157 mCi/mmoll; PerkinElmer Life Sciences) at 30 °C. After labeling, cells were chilled on ice, collected by centrifugation, and suspended in ethanol, water, diethyl ether, pyridine, 15% ammonia (15:15:5:1:0.018, v/v). After a 15-min incubation at 60 °C, cell debris and extracted lipids were separated by a 2-min centrifugation at 2,000 × g and at room tempera-
ture. Radioactivity was measured using a liquid scintillation system (LSC-3600; Aloka, Tokyo, Japan), and samples containing lipids of equal radioactivity were used for further study. Alkaline treatment was performed on each lipid solution by incubating it with a 1:5 volume of 0.5 N NaOH in methanol for 30 min at 37 °C, followed by neutralizing with acetic acid. Lipids were dried and then suspended in 100 μL of water-saturated 1-butanol. To desalt the samples 50 μL of water was added, and the solution was mixed vigorously and then separated into phases by centrifugation. Lipids in the water phase were re-extracted by adding 100 μL of water-saturated 1-butanol. Organic phases were mixed, dried, and suspended in 20 μL of chloroform/methanol/water (5:4:1, v/v). Lipids were separated by TLC on Silica Gel 60 high performance TLC plates (Merck) with chloroform, methanol, 4.2 N ammonia (9:7:2, v/v) or chloroform, methanol, 15 N ammonia (60:12:1, v/v) as the solvent system.

[3H]Inositol labeling was performed on yeast cells grown in YPD medium to ~1.25 × 10^7 cells/mL, and then 1 mL of cells was labeled with 20 μCi of [1,2,3H]inositol (60 Ci/mmol; PerkinElmer Life Sciences) for 1 h at 30 °C. Lipid extraction and TLC separation were done as described above.

**Plasmids**—The plasmid pUG23, a yeast expression vector encoding a fusion protein with a C-terminal enhanced green fluorescent protein under the control of the MET15 promoter, was a gift from Dr. J. H. Hegemann (Heinrich-Heine University, Düsseldorf, Germany). The enhanced green fluorescent protein region of pUG23 was removed altogether or replaced with a 3xFLAG tag, creating the pWK151 or pAK881 plasmid, respectively.

The plasmid pSH14 (PHSI-3xFLAG) was constructed in our laboratory. The PHSI gene was amplified from yeast genomic DNA by PCR using the primers 5’-TTTTCTAGAATTCTAC-

| Mutation | Nucleotide sequence |
|----------|---------------------|
| L12A     | 5’-CACCATGCTTCTGGGACCCCTTTTAATATTTG-3’ |
| Y15A     | 5’-CCCTTCTACCCTGCTTGAATTTGGTGGAAG-3’ |
| N16A     | 5’-CTTACCCCTTTATGTTTCTTCTATTTGGAAG-3’ |
| W23A     | 5’-TTTTCTGTGTTGCTGCTTTTCTATTGCTTTTAC-3’ |
| Q54A     | 5’-GTGCTCACTTTCGTTCTGATAATGTGATGACC-3’ |
| E60A     | 5’-GTGCTCACTTTCGTTCTGATAATGTGATGACC-3’ |
| Q79A     | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| R83A     | 5’-CAGAATTCGGCTAGCACGTATGGTTCTTCAAGAC-3’ |
| W112A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| E116A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| R119A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Y120A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| R141A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Y142A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| F145A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Y149A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Y150A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| G152A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| P188A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Q200A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| R201A    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| N terminus | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| G38/Q    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| G38/Q    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| R70/S    | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| T397/S   | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| G132/A   | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| Q170/Y   | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |
| C terminus | 5’-CTGACCACTTGGAGCGACGTATGGTTCTTCAAGAC-3’ |

**TABLE 1**

| Primers used in this study |
|-----------------------------|
| Only sense primers are presented. |

**Topology and Essential Residues of Phs1**
Modifications. Briefly, yeast spheroplasts in lysis buffer A (20 mM HEPES-NaOH (pH 7.5), 12.5% sucrose, 2 mM MgCl₂, 1× Complete™ protease inhibitor mixture (EDTA-free; Roche Diagnostics), 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) were lysed using an electric Potter homogenizer for 10 strokes. Unlysed cells were removed by centrifugation at 1,300 × g for 3 min, and the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The resulting pellet (total membrane fraction) was suspended in 1 ml of lysis buffer A and loaded onto a step sucrose gradient (0.75 ml of 26% sucrose (w/v), 0.75 ml of 34% sucrose, 1.5 ml of 42% sucrose, 3 ml of 46% sucrose, 2 ml of 50% sucrose, 1.5 ml of 54% sucrose, and 1 ml of 60% sucrose) in buffer (20 mM HEPES-NaOH (pH 7.5), 2 mM MgCl₂, 1× Complete™, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). After centrifugation at 13,000 g for 3 min, the supernatant was centrifuged at 13,000 × g for 20 min at 4 °C, and the resulting pellet (low speed pellet) was suspended in 150 µl of lysis buffer B and then mixed with 850 µl of solubilization buffer (50 mM HEPES-KOH (pH 6.8), 150 mM KOAc, 2 mM Mg(OAc)₂, 1% digitonin, 200 mM sorbitol, 1× Complete™, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). After a 30-min incubation at 4 °C, samples were subjected to ultracentrifugation (100,000 × g) for 30 min at 4 °C. The resulting supernatant was incubated at 4 °C for 2 h with anti-FLAG M2-agarose (Sigma). Beads were washed twice with 1 ml of wash buffer (150 mM HEPES-KOH (pH 6.8), 150 mM KOAc, 2 mM Mg(OAc)₂, 0.5% digitonin, 200 mM sorbitol, and 1 mM dithiothreitol), and bound proteins were eluted three times with 75 µl of elution buffer (wash buffer plus 100 µg/ml 3xFLAG peptide (Sigma)). Elution fractions were pooled and stored at −80 °C.

In vitro 3-hydroxyacyl-CoA dehydratase assay—Yeast spheroplasts (~3 × 10⁸ cells) in lysis buffer B (50 mM HEPES-KOH (pH 6.8), 150 mM KOAc, 2 mM Mg(OAc)₂, 150 mM NaCl, 250 mM sorbitol, 1× Complete™, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) were lysed using an electric Potter homogenizer for 10 strokes. Unlysed cells were removed by centrifugation at 1,300 × g for 3 min. The supernatant was centrifuged at 13,000 × g for 20 min at 4 °C, and the resulting pellet (low speed pellet) was suspended in 150 µl of lysis buffer B and then mixed with 850 µl of solubilization buffer (50 mM HEPES-KOH (pH 6.8), 150 mM KOAc, 2 mM Mg(OAc)₂, 1% digitonin, 200 mM sorbitol, 1× Complete™, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). After a 30-min incubation at 4 °C, samples were subjected to ultracentrifugation (100,000 × g) for 30 min at 4 °C. The resulting supernatant was incubated at 4 °C for 2 h with anti-FLAG M2-agarose (Sigma). Beads were washed twice with 1 ml of wash buffer (150 mM HEPES-KOH (pH 6.8), 150 mM KOAc, 2 mM Mg(OAc)₂, 0.5% digitonin, 200 mM sorbitol, and 1 mM dithiothreitol), and bound proteins were eluted three times with 75 µl of elution buffer (wash buffer plus 100 µg/ml 3xFLAG peptide (Sigma)). Elution fractions were pooled and stored at −80 °C.

In vitro IPC synthase assay—In vitro IPC synthase assays were performed by mixing purified Phs1–3xFLAG protein in reaction buffer (total volume of 50 µl; 150 mM HEPES-KOH (pH 6.8), 2 mM Mg(OAc)₂, 0.5% digitonin, and 1 mM dithiothreitol) with 0.05 µCi of 3-[¹⁴C]hydroxypalmitoyl-CoA (55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO) and then incubating the mixture at 37 °C for various times. The reactions were terminated by adding 25 µl of 75% KOH (w/v) and 50 µl of ethanol, then saponified at 70 °C for 1 h, and acidified by adding 100 µl of 5 N HCl with 50 µl of ethanol. Lipids were extracted twice, each with 700 µl of hexane, and then the extracts were pooled, dried, and suspended in 35 µl of chloroform. Lipids were separated by TLC on LK50F Silica Gel 150A TLC plates (Whatman, Kent, UK) with hexane/diethyl ether/acetone acid (30:70:1, v/v) as the solvent system.

Immunoprecipitation—Yeast spheroplasts in lysis buffer C (50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 250 mM sorbitol, 1× Complete™, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) were lysed by sonication then treated with 1% digitonin. Proteins were immunoprecipitated using anti-FLAG M2-agarose, separated by SDS-PAGE, and subjected to immunoblotting as described above.

RESULTS

Decreases in Phs1 Levels Cause a Significant Accumulation of PHS with C20 Chain Length—Previous studies found that decreases in Phs1 cause an accumulation of LCβs (18, 19), yet quantitative analyses have not been performed. Therefore, we investigated this using HPLC. Because the PHS1 gene cannot be deleted because of its essential function, we used the yeast strain TH_3237 and its derivative (referred to here as Tet-PHS1), which carries the PHS1 gene under the control of the TetO₅ promoter. The Tet-PHS1 cells were unable to form colonies on YPD plates containing doxycycline (DOX), which shuts off gene expression under the TetO₅ promoter (data not shown).
Tet-PSH1 cells was only slightly slower than that of the wild type cells (Fig. 2A). Thus, to avoid nonspecific effects caused by growth inhibition, we studied DOX-treated Tet-PSH1 cells at earlier time points (4–6 h after adding DOX).

HPLC analysis of wild type cells cultured in YPD medium revealed the major cellular LCBS produced to be PHS18 (PHS with C18 chain length) and DHS18, followed by DHS16 (Fig. 2, B and C). In the Tet-PSH1 cells, all three of these LCBS were significantly increased compared with those in wild type cells, even in the absence of DOX (Fig. 2C). Moreover, PHS20, which was barely detected in wild type cells cultured in YPD medium, was found at high levels in the Tet-PSH1 cells, levels even greater than those of DHS18 and DHS16 (Fig. 2, B and C). When DOX was added to the medium, PHS18, DHS18, and DHS16 increased slightly in the Tet-PSH1 cells, and the increase in PHS20 was more prominent (Fig. 2C). These results suggest that C18-CoA, the precursor of PHS20, accumulates in Tet-PSH1 cells.

The accumulation of LCBS observed in the Tet-PSH1 cells not treated with DOX may have been because of weaker expression of Phs1 from the TetO₃ promoter compared with the natural PHS1 promoter. We tagged the endogenous PHS1 gene with 3xFLAG and examined gene expression. We estimated that the Tet-PSH1 cells expressed ~1:20 the amount of Phs1 protein compared with wild type cells (data not shown). These results indicate that such a decrease in Phs1 levels does affect the cellular VLCFA levels, as well as the levels of LCFA and LCB, but still supports nearly normal cell growth.

Decreases in Phs1 Levels Cause an Accumulation of CER and a Reduction in Complex Sphingolipids—To examine the effect of decreased Phs1 levels on sphingolipid metabolism, we labeled wild type and Tet-PSH1 cells with [¹⁴C]Ser in the presence of DOX. [¹⁴C]Ser was incorporated into phosphatidylserine, converted to phosphatidylethanolamine, and further to phosphatidicholine (Fig. 3A). Alkaline treatment abolished the labeled glycerophospholipids by hydrolyzing ester linkages (Fig. 3, A and B). The amounts of labeled phosphatidylserine, phosphatidylethanolamine, and phosphatidicholine were similar between the wild type and Tet-PSH1 cells, but sphingolipid synthesis was greatly affected in the Tet-PSH1 cells. DHS, PHS, and CER were all increased, whereas IPC, mannosylinositol phosphorylceramide (MIPC), and mannosylinositol phosphophorylceramide (M(IP)₂C) were decreased (Fig. 3, A and C). CER accumulation was the most prominent. These results indicate that CER-to-IPC conversion is largely affected by a reduction in Phs1 levels. Furthermore, when labeled sphingolipids were separated by TLC using another solvent system, it became apparent that the CER species differed between the wild type and Tet-PSH1 cells (Fig. 3B). In wild type cells the most abundant CER contains α-hydroxy-C26 FA and PHS (20). However, the most prominent band in the Tet-PSH1 cells migrated more slowly on the TLC, i.e. was more hydrophilic, compared with the CER band from the wild type cells. The more hydrophilic band is likely a CER with α-hydroxy-C16 FA and PHS, considering that another VLCFA elongation-deficient cell line, Δybrr159w, is known to accumulate this CER (20). The amount of this putative CER carrying α-hydroxy-C16 FA and PHS was 2.3-fold higher in the Tet-PSH1 cells than the amount of CER.

**FIGURE 2.** Decreased Phs1 levels cause an accumulation of LCBS. A, SAY31 (wild type, WT) and SAY32 (Tet-PSH1, Tet) cells were grown at 30 °C in YPD medium in the presence or absence of 10 μg/ml DOX. At the indicated time points following the addition of DOX to the culture media, aliquots of cell suspensions were measured for cell density (A₅₉₀) as a determination of growth. Values represent the mean ± S.D. from three independent experiments. B and C, R1158 (wild type) and SAY33 (Tet-PSH1) cells were grown for 6 h at 30 °C in YPD medium in the presence (B and C) or absence (C) of 10 μg/ml DOX. Lipids were extracted, treated with o-phthalaldehyde, and analyzed by reverse-phase HPLC. The area of each peak representing an LCB was quantified using sphingosine with C18 chain length (SPH₁₈) as an internal control and is presented in C. Values represent the mean ± S.D. from three independent experiments.
carrying α-hydroxy-C16 FA and PHS found in wild type cells. Other CER species present in the Tet-PHS1 cells (Fig. 3B, upper bands) likely also correspond to those found in Δybr159w cells, including CER with nonhydroxy-C16 FA and PHS (20).

We also labeled the wild type and Tet-PHS1 cells with [3H]inositol. In the wild type cells a significant amount of PI was converted to complex sphingolipids (IPC, MIPC, and M(IP)2C) and PI monophosphates (phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate) (Fig. 4). Synthesis of these complex sphingolipids was again reduced in the Tet-PHS1 cells, and addition of DOX furthered the reduction (Fig. 4). Compared with the reduction in MIPC, reductions in IPC and M(IP)2C were much more pronounced. Conversely, in the Tet-PHS1 cells the lyso-PI levels were increased (Fig. 4), something often observed in sphingolipid synthesis-deficient cells (31), although the molecular mechanism of its production is not known. The amount of labeled PI was also slightly increased compared with that in wild type cells, probably because of a block of further metabolism (Fig. 4). Noticeably, the production of PI monophosphates was also decreased in the Tet-PHS1 cells (Fig. 4) (see “Discussion”).

Although CER-to-IPC conversion was greatly affected in the Tet-PHS1 cells, any causative mechanism was unclear. CER is synthesized in the ER and transported to the Golgi, where it receives a phosphoinositol moiety transferred from PI by the PI:CER phosphoinositol transferase (IPC synthase) Aur1, generating IPC. Aur1 is an integral membrane protein, so if it is synthesized in the ER it must also be transported to the Golgi. One possible mechanism causing the impairment in CER-to-IPC conversion is an indirect effect by the defective VLCFA synthesis on the localization or activity of Aur1. To examine the intracellular localization of Aur1, we performed a sucrose gradient fractionation. The ER and the Golgi were nicely separated by this fractionation (Fig. 5A). However, the activity of Aur1 and its Golgi localization were indistinguishable between the wild type and Tet-PHS1 cells (Fig. 5B). Therefore, the accumulation of CER in Tet-PHS1 cells is because of something other than decreased activity or mislocalization of this enzyme (see also “Discussion”).

Tyr-149 and Glu-156 Residues Are Essential for Phs1 Function—The Phs1 family is conserved among eukaryotes. To begin to identify residues in Phs1 family members essential to its activity, we first compared amino acid sequences of 31 family members from 24 organisms and found that 22 amino acid residues are conserved (supplemental Fig. S1). We changed
R83A, R141A, Y149A, G152A, and E156A mutants could not support cell growth. The amount of Phs1 was evaluated by immunoblotting with antibodies against Phs1–3xFLAG and Sec61, a marker for the ER. Gln-79, Arg-83, and Arg-141 were important, and Gly-152 and Tyr-149 were essential for the function of Phs1. Glu-156 and Arg-119 were important, and Gly-152 and Tyr-149 were essential for the function of Phs1.

DISCUSSION

Phs1 belongs to a novel 3-hydroxyacyl-CoA dehydratase protein family and is conserved among eukaryotes. Members of this family are involved in the degradation of fatty acids in the peroxisome. The Phs1 protein is a membrane enzyme that traverses the membrane six times and is involved in the degradation of fatty acids in the peroxisome. The essential residues of Phs1 are Tyr-149 and Glu-156, which are directly involved in the catalytic reaction. The catalytic reaction of Phs1 is a time-dependent process, and the presence of these essential residues is necessary for the catalytic activity of Phs1. The results of the in vitro assays indicate that Phs1 belongs to a novel 3-hydroxyacyl-CoA dehydratase family and is conserved among eukaryotes.
Topology and Essential Residues of Phs1

This family exhibit no significant sequence similarity to 3-hydroxyacyl-ACP dehydratases or similar functional domains of FASs, and information regarding its active site and catalytic mechanism has been completely lacking. In the present study we identified several amino acid residues (Gln-79, Arg-83, Arg-141, Tyr-149, Gly-152, and Glu-156) that are important for Phs1 function. In particular, Tyr-149 and Glu-156 are essential for Phs1 activity, because substitution of either residue resulted in a loss of growth restoration of Tet-Phs1 cells (Fig. 6A) and an absence of enzyme activity (Fig. 6D). We also examined the membrane topology of Phs1 and propose that it spans the membrane six times and that its N terminus and C terminus are located in the cytosol (Fig. 7B). In this topology model, the six important residues above are located within or near transmembrane regions 3 and 5. Transmembrane region 5 is particularly important because four important residues, including the two essential residues Tyr-149 and Glu-156, are located within or nearby.

In the 3-hydroxyacyl-ACP dehydratases (or domains) of FASs, His and Asp/Glu residues are essential for catalysis, and their reaction mechanism has been proposed. For instance, in the bacterial 3-hydroxyacyl-ACP dehydratase FabA, the His-70 residue acts as a catalytic base to abstract a proton from the C-2 of 3-hydroxyacyl-ACP, and the Asp-84 residue promotes the removal of the hydroxyl group at C-3 (17). Furthermore, Cys-80, four residues before the Asp-84, is also important as it interacts with the hydroxyl group at C-3 of 3-hydroxyacyl-ACP and that in H2O, before and after the catalysis, respectively, via hydrogen bonds (17). The sole His residue in Phs1, His-196, is not conserved, and in fact an Ala mutant of this residue restored the growth defect of the Tet-Phs1 cells, indicating that this residue is not important for enzyme activity (data not shown). Therefore, we surmised that another amino acid residue must serve as the catalytic base. The deprotonated form of Tyr is one proton-accepting amino acid residue. For example, a Tyr residue acts as one of a catalytic triad in members of the short-chain dehydrogenase reductase superfamily, including the 2,3-butanediol dehydrogenase (18).

Fig. 6. Tyr-149 and Glu-156 are essential for Phs1 activity. SAY32 (Tet-Phs1; Tet) cells bearing pUG23 (vector), pSH14 (wild type Phs1–3xFLAG; WT), pSH15 (L12A), pSH16 (Y15A), pSH2 (W12A), pSH17 (Q54A), pSH18 (E60A), pSH19 (Q79A), pSH20 (R83A), pSH21 (W12A), pSH22 (E156A), pSH23 (R119A), pSH24 (Y120A), pSH25 (R141A), pSH26 (Y142A), pSH27 (Y149A), pSH28 (R150A), pSH30 (G152A), pSH31 (E156A), pSH32 (Y15A), pSH33 (P150A), or pSH34 (R201A) were used. A, cells were grown for 48 h at 30 °C on plates of SC medium lacking His and Met but containing 10 μg/ml DOX. Lipids were extracted and derivatized with o-phthalaldehyde, and samples equivalent to 2.8 x 10^7 cells were analyzed by reverse-phase HPLC, and the area of the peak representing Phs1 was quantified. Each value represents the Phs1 amount relative to that in SAY32 cells expressing wild type Phs1–3xFLAG and presents the mean ± S.D. from three independent experiments. Statistically significant differences are indicated (*, p < 0.05; **, p < 0.01; t test). C, total lysates from B were prepared, and equal amounts of proteins (5 μg) were subjected to immunoblotting with anti-FLAG antibodies. Uniform protein loading was demonstrated by immunoblotting with anti-Pgk1 antibodies. Phs1–3xF, Phs1–3xFLAG. D, using anti-FLAG M2-agarose, Phs1–3xFLAG proteins were affinity-purified from SAY32 (Tet-Phs1) cells bearing pUG23 (vector), pSH14 (wild type Phs1–3xFLAG), pSH28 (Y149A), or pSH31 (E156A). Cells had been grown for 6 h at 30 °C in SC medium lacking His and Met but containing 10 μg/ml DOX. Purified Phs1–3xFLAG proteins (wild type, 1 ng; Y149A and E156A, 4 ng) or mock enzyme solution were incubated with [14C]3-hydroxypalmitoyl-CoA for the indicated times. After termination of the reactions, lipids were saponified, acidified, extracted, separated by TLC, and visualized by autoradiography. 2-Oct-16:1, 2-hydroxyacyl-16:1, 2,3-hexadecadienoic acid. 

FIGURE 6. Tyr-149 and Glu-156 are essential for Phs1 activity. SAY32 (Tet-Phs1; Tet) cells bearing pUG23 (vector), pSH14 (wild type Phs1–3xFLAG; WT), pSH15 (L12A), pSH16 (Y15A), pSH60 (N16A), pSH61 (W23A), pSH7 (Q54A), pSH18 (E60A), pSH19 (Q79A), pSH20 (R83A), pSH21 (W12A), pSH22 (E156A), pSH23 (R119A), pSH24 (Y120A), pSH25 (R141A), pSH26 (Y142A), pSH27 (Y149A), pSH28 (R150A), pSH29 (P150A), or pSH30 (G152A), pSH31 (E156A), pSH63 (P188A), pSH64 (Q200A), or pSH32 (R201A) were used. A, cells were grown for 48 h at 30 °C on plates of SC medium lacking His and Met in the presence of 0 μg/ml DOX, and growth was determined visually. Symbols indicate the following: −, no growth; ±, slow growth; and +, normal growth, compared with SAY32 cells expressing wild type Phs1–3xFLAG. B, cells were grown at 30 °C for 6 h in SC medium lacking His and Met but containing 10 μg/ml DOX. Lipids were extracted and derivatized with o-phthalaldehyde. Samples equivalent to 2.8 x 10^7 cells were analyzed by reverse-phase HPLC, and the area of the peak representing Phs1 was quantified. Each value represents the Phs1 amount relative to that in SAY32 cells expressing wild type Phs1–3xFLAG and presents the mean ± S.D. from three independent experiments. Statistically significant differences are indicated (*, p < 0.05; **, p < 0.01; t test). C, total lysates from B were prepared, and equal amounts of proteins (5 μg) were subjected to immunoblotting with anti-FLAG antibodies. Uniform protein loading was demonstrated by immunoblotting with anti-Pgk1 antibodies. Phs1–3xF, Phs1–3xFLAG. D, using anti-FLAG M2-agarose, Phs1–3xFLAG proteins were affinity-purified from SAY32 (Tet-Phs1) cells bearing pUG23 (vector), pSH14 (wild type Phs1–3xFLAG), pSH28 (Y149A), or pSH31 (E156A). Cells had been grown for 6 h at 30 °C in SC medium lacking His and Met but containing 10 μg/ml DOX. Purified Phs1–3xFLAG proteins (wild type, 1 ng; Y149A and E156A, 4 ng) or mock enzyme solution were incubated with [14C]3-hydroxypalmitoyl-CoA for the indicated times. After termination of the reactions, lipids were saponified, acidified, extracted, separated by TLC, and visualized by autoradiography. 2-Oct-16:1, 2-hydroxyacyl-16:1, 2,3-hexadecadienoic acid. 

DEY113 cells bearing pSH86 (YBR159w-3xHA) were transfected with pW1K151 (vector), pSH14 (wild type Phs1–3xFLAG), pSH28 (Y149A), or pSH31 (E156A). Cells were grown for 6 h at 30 °C in SC medium lacking His, Met, and uracil. Total cell lysates were prepared from the cells and solubilized with 1% digitonin. Following immunoprecipitation with anti-FLAG M2 agarose, bound material and input fractions were subjected to immunoblotting with anti-FLAG or anti-HA antibodies. WT, wild type; IP, immunoprecipitation; IB, immunoblotting.
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FIGURE 7. Phs1 is a membrane protein that traverses the membrane six times with its N terminus and C terminus exposed to the cytosol. A, total lysates were prepared from DEY102 cells bearing pSH14 (PHS1-3xFLAG without insertion), pSH69 (N terminus; N-term), pSH38 (G38/Q; insertion between Gly-38 and Gin-39), pSH39 (R70/S), pSH40 (T97/S), pSH41 (G132/A), pSH42 (Q170/Y), or pSH70 (C-term). Lysates were untreated or treated with Endo H and separated by SDS-PAGE, followed by immunoblotting with anti-FLAG antibodies. B, model for the topology of Phs1 is illustrated. Gray circles indicate amino acid residues conserved among 31 Phs1 homologs. Amino acid residues whose mutation resulted in weak or no growth in Fig. 6A are illustrated as black circles and squares, respectively. Arrowheads indicate the insertion sites of the glycosylation cassette for the topology assay.

dehydrogenase/reductase family, and its deprotonated form functions as the proton acceptor (34). We found only two amino acids (Tyr-149 and Glu-156) essential for the function of Phs1 (Fig. 6A), so we hypothesize that these two residues constitute an active site that functions in the abstract of a proton from the C-2 of 3-hydroxyacyl-CoA and in the removal of the hydroxyl group at C-3, respectively. Analogous to the Cys-80 of FabA, the Gly-152 of Phs1, which is also located four residues before the Glu-156, may interact with the hydroxyl groups at C-3 of 3-hydroxyacyl-CoA and in H2O, prior to and after the catalysis, respectively. It is possible that the Gin-79 and Arg-83 residues in transmembrane region 3 are positioned near the Tyr-149, Gly-152, and Glu-156 in transmembrane region 5 in the folded state, and that Arg-83 stabilizes the deprotonated state of Tyr-149. However, in our topology model the putative active site (comprising Tyr-149, Gly-152, and Glu-156) is located in the interior of the membrane. It is also possible that the substrate 3-hydroxyacyl-CoA is deeply embedded in the membrane during the reaction. Consistent with this notion, a previously reported biochemical study investigating the sensitivity of rat liver microsomal membranes to several proteases and observations with an anti-3-hydroxyacyl-CoA dehydratase antibody suggested that the active site of the mammalian enzyme is embedded in the microsomal membrane, in contrast to those of the condensing enzyme and 2,3-trans enoyl-CoA reductase (35). Alternatively, the prediction of the transmembrane region is incorrect, and the location of the putative active site is actually more proximal to the cytosolic surface.

To further determine the role of VLCFAs in sphingolipid metabolism, we investigated the effect of exogenous C26 FA (20 μM) on the Tet-PHS1 cells. Exogenous C26 FA had almost no effect on the growth of the cells (data not shown). LCB levels did decrease upon addition of C26 FA, but only slightly, with the amount of PHS18 in the Tet-PHS1 cells decreasing from 940 pmol/10^7 cells in the absence of C26 FA to 770 pmol/10^7 cells in its presence, still much higher than levels in wild type cells (18.7 pmol/10^7 cells). [14C]Ser labeling demonstrated no apparent change in the sphingolipid pattern after the addition of the C26 FA (data not shown). Thus, exogenous C26 FA was not able to be utilized by these cells. Several reasons for this are conceivable. The most likely possibility we considered is low efficiency of the conversion of C26 FA to C26-CoA. Indeed, cellular very long-chain fatty acyl-CoA synthetase activity in yeast cells is significantly lower than long-chain fatty acyl-CoA synthase activity (36). Yeast does contain the very long-chain fatty acyl-CoA synthetase Fat1, which is specific for substrates with acyl chains C20 and longer (36), as well as five other yeast acyl-CoA synthetases with other specificities (37). In contrast to exogenously added VLCFAs, endogenous very long-chain fatty acyl-CoA synthetases are synthesized by the VLCFA elongation system, mainly from C16-CoA produced by FAS without the release of free VLCFAs. Another possibility regarding lack of effect of exogenous C26 FA is difficulty in importing C26 FA or in transporting it from the plasma membrane to the ER. Indeed, an absence of phenotypic reversion following treatment with exogenous VLCFA has been reported for another mutant defective in VLCFA synthesis, Δybr159w (38).

Of the steps in sphingolipid biosynthesis, the step most affected by a reduction in Phs1 levels is the CER-to-IPC conversion, although a causative mechanism is unclear. CER with a shorter chain length was accumulated in the Tet-PHS1 cells (Fig. 3B). It is possible that the IPC synthase Aur1 exhibits low activity toward such short-chain CER. However, another possibility may be more likely, because Aur1 efficiently converts even shorter C6-7-nitrobenz-2-oxa-1,3-diazol-4-yl-CER to IPC in vivo and in vitro (30). More convincing, however, is that the localization and activity of Aur1 were not affected in the Tet-PHS1 cells (Fig. 5B). We speculate then that the transport of CER and/or PI is impaired in the Tet-PHS1 cells. Both CER and
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PI are synthesized in the ER and delivered to the Golgi for conversion to IPC. However, their transport mechanism in yeast is not known, although in mammals the transport of CER occurs by the transfer protein CERT (39). Consistent with the theory of impaired transport, the production of both complex sphingolipids and PI monophosphates, which also requires transport from the ER (40), was affected in the Tet-PHS1 cells (Figs. 3 and 4). Moreover, in $[^3]$H]inositol labeling experiments the CER-to-IPC and MIPC-to-M(IP)2C steps, both of which require supply of PI, were more affected than the IPC-to-MIPC step in the Tet-PHS1 cells (Fig. 4). These results suggest that PI transport is indirectly impaired by deficient VLCFA synthesis.

Mammals have two Phs1 homologs, PTPLA and PTPLB, although their functions remain unclear. Forced expression of PTPLA or PTPLB in the Tet-PHS1 yeast cells rescued the growth in the presence of DOX, indicating that both proteins possess functions identical to Phs1, i.e. 3-hydroxyacyl-CoA dehydratase activity. Disruption of the PTPLA gene in dogs causes myotubular (centronuclear) myopathy (23). Interestingly, mutations in the MTM1 gene encoding myotubulin also result in this disease in humans (41). Myotubulin catalyzes the dephosphorylation of phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate (42–44). Thus, altered metabolism of phosphoinositides may be one cause of this myopathy. It is possible that disruption of the PTPLA gene also affects the phosphoinositide metabolism as described above for Phs1, leading to myotubular myopathy.

Fen1/Sur4, Ybr159w, Phs1, and Tsc13 form elongase complex(es) (18). In addition to the VLCFA elongation, this complex seems to function in or couple with other cellular processes by interacting with other proteins. For example, Tsc13 interacts with Nyv1, which is involved in the formation of nucleus-vacuole junctions (45). Nucleus-vacuole junctions are sites of piecemeal microautophagy of the nucleus, during which nonessential portions of the nucleus are pinched off into invaginations of the vacuole membrane and then degraded in the vacuole lumen (46). VLCFA s have been proposed to be required for the efficient biogenesis of the highly curved blebs and vesicles observed during the microautophagy of the nucleus by promoting the formation of highly curved membrane structures (45). In addition to Nyv1, the Fen1/Sur4, Ybr159w, Phs1, and Tsc13 complex seems to interact with numerous proteins. Comprehensive protein-protein interaction analyses, by affinity capture and a yeast two-hybrid method, determined that proteins in this complex interact with more than 100 proteins (47–49). These included proteins involved in lipid metabolism such as ergosterol synthesis (Erg3, Erg11, and Erg25), sphingolipid metabolism (Csg2, Lac1, Ypc1, Ydc1, and Sur2), and monounsaturated fatty acid synthesis (Ole1), and in protein transport from the ER to the Golgi (Emp24, Emp46, Emp47, Erp4, Erp5, Erv14, Erv29, Erv41, and Yop1). Although these interactions must be confirmed by other methods such as co-immunoprecipitation, it is possible that Fen1/Sur4, Ybr159w, Phs1, and Tsc13 might form extremely large complex(es) functioning or coupling with other cellular processes. To coordinate VLCFA synthesis and sphingolipid synthesis, it is possible that CERT and/or PI transport is regulated by this hypothesized large complex. However, future studies are required to determine the details of any such complex.

Acknowledgments—We thank Dr. R. Schekman for providing anti-Secl1 antibodies, Dr. S. Munro for anti-Atg1 antibodies, and Dr. J. H. Hegemann for the pUG23 plasmid. We are grateful to Dr. E. A. Sweeney for scientific editing of the manuscript.

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