Regulation of the Sphingoid Long-chain Base Kinase Lcb4p by Ergosterol and Heme

STUDIES IN PHYTOSPHINGOSINE-RESISTANT MUTANTS

Received for publication, March 22, 2005, and in revised form, August 31, 2005 Published, JBC Papers in Press, September 1, 2005, DOI 10.1074/jbc.M503147200

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Sphingoid long-chain base 1-phosphates (LCBPs) are widely conserved, bioactive lipid molecules. In yeast, LCBPs are known to be involved in several cellular responses such as heat shock resistance and Ca\(^{2+}\) mobilization, although their target molecules and signaling pathways remain unclear. To identify genes involved in LCBP signaling and in regulation of LCBP synthesis, we performed transposon mutagenesis in cells lacking the LCBP lyase DPL1 and LCBP phosphatase LCB3 genes and screened for phytosphingosine-resistant clones. Further isolation and identification revealed eight genes (PBP1, HEM14, UFDF4, HMG1, TPS1, KES1, WHI2, and ERG5), in addition to the previously characterized LCB4 and PDR5 genes, that are involved in phytosphingosine resistance. Of these eight, four are ergosterol-related genes (HEM14, HMG1, KES1, and ERG5). We also demonstrated that protein expression of the long-chain base kinase Lcb4p was reduced in Δhem14 and Δhmg1 cells, likely as a consequence of decreased activity of the heme-dependent transcription factor Hap1p. In addition, phosphorylation of Lcb4p was decreased in all the ergosterol-related mutants isolated and other ergosterol mutants constructed (Δerg2, Δerg3, and Δerg6). Finally, plasma membrane localization of Lcb4p was found to be reduced in Δerg6 cells. These results suggest that changes in sterol composition affect the phosphorylation of Lcb4p because of the altered localization. The other genes isolated (PBP1, UFDF4, TPS1, and WHI2) may be involved in LCBP signaling.

Sphingolipids are major membrane components of eukaryotic cells. Ceramide, the backbone of sphingolipids, is formed by the amide linkage of a sphingoid long-chain base (LCB)\(^2\) with a fatty acid. The major mammalian LCB is sphingosine, whereas in the yeast *Saccharomyces cerevisiae*, dihydrophosphoglucone (DHS) and phytosphingosine (PHS) serve as LCBs. LCBs are bioactive lipid molecules involved in apoptosis, endocytosis, and G\(_1\) cell cycle arrest (1–3). The phosphorylation of LCBs at C-1 results in the production of long-chain base 1-phosphates (LCBPs), sphingosine 1-phosphate (SIP) in mammals and PHS 1-phosphate (PHS1P) and DHS 1-phosphate in yeast. Interestingly, LCBPs possess completely different biological functions compared with the original LCBs.

In mammalian cells, SIP is known to elicit various cellular responses via the cell-surface SIP/Edg (endothelial differentiation gene) receptors, such as proliferation, motility, differentiation, and immunity (4–7). In addition to its role as an extracellular signal mediator, SIP also functions intracellularly. Intracellular SIP has been proposed to be involved in Ca\(^{2+}\) mobilization, cell proliferation, G\(_1\)/S cell cycle transition, and inhibition of apoptosis (4–7), although its intracellular target molecules and signaling pathways remain unclear.

Because yeast cells do not possess a cell-surface receptor for LCBP/SIP, their LCBPs function only intracellularly (8–11). Yeast LCBPs and mammalian intracellular SIP share some effects. For instance, both influence Ca\(^{2+}\) mobilization. Similarly, LCBPs confer protection from environmental stresses, as in the inhibition of apoptosis in mammalian cells and heat resistance in yeast. Such shared functions imply evolutionarily conserved target molecules and signaling pathways for LCBPs.

The enzymes responsible for both the production and degradation of LCBPs are, in fact, completely conserved from yeast to mammals. In mammalian cells, two known sphingosine kinases, SPHK1 and SPHK2, produce SIP; in yeast cells, two LCB kinases, Lcb4p and Lcb5p, which share significant homology with SPHK1 and SPHK2, synthesize LCBPs. In turn, LCBPs are degraded either back to LCBs by the mammalian SPPL and SPP2 or yeast Lcb3p and Ysr3p phosphatases or to fatty aldehyde and phosphoethanolamine by mammalian SPL or yeast Dpl1p lyase.

Because LCBPs are signaling molecules, their intracellular levels must be strictly regulated. However, knowledge about the regulatory mechanism(s) of their synthesis and degradation is still limited. The activity and/or localization of mammalian SPHK1 is known to be regulated by its association with other proteins (12–15) or by its phosphorylation by ERK1/2 (extracellular signal-regulated kinase-1/2) (16). Likewise, we recently found that the stability of yeast Lcb4p is regulated through its phosphorylation by the cyclin-dependent protein kinase Pho85p via a ubiquitin-dependent pathway (17).

In this study, we investigated the LCBP signaling pathways as well as the regulatory mechanism(s) of LCBP synthesis by screening transposon-inserted mutants of a PHS-resistant yeast cell. Identification of the sites of transposon insertion revealed that, in addition to the previously described genes LCB4 and PDR5, eight other genes are involved in PHS resistance. In mutants of four ergosterol-related genes (HEM14, HMG1, KES1, and ERG5), the amount and/or phosphorylation of Lcb4p was reduced. Additional analyses suggested that dysfunction of heme synthesis and altered composition of ergosterol are related to the decreased amount and phosphorylation of Lcb4p, respectively.

**MATERIALS AND METHODS**

*Yeasts and Strains*—The yeast strains used in this study are listed in TABLE ONE. Cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30 °C. The Δlcb3::URA3 cells were constructed by replacing the 0.8-kb BamHI-Hpal region in the
TABLE ONE

Yeast strains used in this study

| Strain        | Genotype                        | Source      |
|---------------|---------------------------------|-------------|
| SEY6210       | MATa leu2-3,112 ura3-52 his3Δ200 trp1-Δ901 lys2-801 suc2-Δ9 | Ref. 49     |
| KHY13         | SEY6210, Δdp1::TRP1             | Ref. 20     |
| KHY17         | SEY6210, Δdp1::TRP1 Δsep4::LEU2 | This study  |
| KHY22         | SEY6210, Δdp1::TRP1 Δlcb3::LEU2 | Ref. 19     |
| KHY360        | SEY6210, Δdp1::TRP1 LEU2        | Ref. 19     |
| KHY364        | KHY360, Δpdr5::KanMX4           | Ref. 19     |
| KHY388        | SEY6210, Δdp1::TRP1 Δlcb3::URA3 | This study  |
| KHY389        | SEY6210, Δdp1::TRP1 Δlcb3::URA3 sup | This study |
| KHY406        | KHY389, LEU2                    | This study  |
| KHY420        | KHY389, Δerg6::KanMX4           | This study  |
| KHY479        | KHY389, Δphp1::KanMX4           | This study  |
| KHY480        | KHY389, Δwhi2::KanMX4           | This study  |
| KHY481        | KHY389, Δufd4::KanMX4           | This study  |
| FKY47         | KHY389, Δhem14::KanMX4          | This study  |
| FKY48         | KHY389, Δerg5::KanMX4           | This study  |
| TS14          | KHY389, Δlcb4::KanMX4           | This study  |
| TS33          | KHY389, Δlcb4::KanMX4           | This study  |
| TS34          | KHY389, Δlcb4::KanMX4           | This study  |
| TS45          | KHY389, Δkes1::KanMX4           | This study  |
| TS114         | KHY389, Δerg2::URA3             | This study  |
| TS115         | KHY389, Δerg3::HIS3             | This study  |
| TS116         | KHY389, Δerg2::URA3 Δerg5::KanMX4 | This study |
| TS117         | KHY389, Δerg3::HIS3 Δerg5::KanMX4 | This study |
| TS122         | KHY389, Δleur1::LEU2            | This study  |
| TIY1          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY2          | KHY389, php1::mTn-lacZ::LEU2    | This study  |
| TIY3          | KHY389, hem14::mTn-lacZ::LEU2   | This study  |
| TIY4          | KHY389, ufd4::mTn-lacZ::LEU2    | This study  |
| TIY5          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY6          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY7          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY8          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY9          | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |
| TIY10         | KHY389, lcb4::mTn-lacZ::LEU2    | This study  |

LCB3 gene with the URA3 marker. The Δnap1::LEU2 cells were constructed by replacing the 1.7-kb HindIII-MscI region in the HAPI gene with the LEU2 marker. The Δlcb4::KanMX4, Δphp1::KanMX4, Δhem14::KanMX4, Δufd4::KanMX4, Δlcb4::KanMX4, Δkes1::KanMX4, Δlcb4::KanMX4, Δkes1::KanMX4, Δues1::KanMX4, Δerg6::KanMX4, and Δerg3::HIS3 cells were constructed by replacing their entire open reading frames with the respective markers. Standard genetic methods were performed as described (18).

Isolation of PHS-resistant Yeast Mutants—Mutagenesis by random insertion of the transposon mTn-lacZ::LEU2 was performed as described previously (19) using a yeast genomic library kindly provided by Dr. Michael Snyder (Yale University, New Haven, CT). The library was digested with NotI, and the resulting DNA fragments were transformed into the KHY389 (Δdp1::Δlcb3) cells. Pooled transposon-inserted mutants were plated at a density of 1 × 10^5 cells/plate on YPD medium containing 7.5 μM PHS and 0.015% Nonidet P-40 as a dispersant. The plates were then incubated at 30 °C for 3 days. PHS-resistant yeast mutants were obtained at a frequency of ~1/3600 mutants. We randomly selected 36 PHS-resistant mutants and subjected these to additional analyses. The sites of transposon insertion in the isolated mutants were identified according to the recommendations of the Yale Genome Analysis Center (available at ygac.med.yale.edu/).

Immunofluorescence Microscopy—Cells were fixed with formaldehyde, converted to spheroplasts, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin as described previously (20). The cells were then incubated with 1 μg/ml 4',6-diamidino-2-phenylindole (Roche Applied Science, Mannheim, Germany) in phosphate-buffered saline for 20 min at room temperature. Cells were washed, mounted on glass slides using a SlowFade antifade kit (Molecular Probes, Inc., Eugene, OR), and observed under a fluorescence microscope (Axioskop 2 plus, Carl Zeiss AG, Oberkochen, Germany).

Biochemical and Immunochemical Studies—Cellular uptake of [4,5-^3H]DHS (American Radiolabeled Chemicals, St. Louis, MO) was assessed by TLC as described previously (19). Radioactivity associated with DHS was quantified using NIH Image Version 1.62 software.

Immunoblotting was performed as described previously (20), and labeling was detected with ECL™ Western blot detection reagents (Amersham Biosciences). Affinity-purified anti-Lcb4p antibodies (1:1000 dilution) (17), anti-Pgk1p antibodies (0.0625 μg/ml Molecular Probes, Inc.), anti-Dpm1p antibodies (2 μg/ml Molecular Probes, Inc.).
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TABLE TWO

Accumulation of LCBPs inhibits cell growth

| Strain                  | PHS 0 μM | PHS 5 μM | PHS 7.5 μM | PHS 10 μM | PHS 12.5 μM | PHS 15 μM | PHS 20 μM |
|-------------------------|----------|----------|------------|-----------|-------------|-----------|-----------|
| SEY6210                 | +++      | +++      | +          | +/−       | −           | −         | −         |
| SEY6210/pRS314/pRS315   | +++      | +++      | +          | +/−       | +           | +/−       | +/−       |
| KHY13/pRS315            | +++      | +++      | +          | +/−       | −           | −         | −         |
| KHY22                   | +++      | +++      | +          | +/−       | −           | −         | −         |
| KHY388                  | +/−       | +/−       | −          | −         | −           | −         | −         |
| KHY389                  | +        | +/−       | −          | −         | −           | −         | −         |

*Cells exhibited heterogeneous growth.

and anti-Pma1p antibodies (0.4 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary antibodies. Peroxidase-conjugated donkey anti-rabbit IgG (γ-H270, fragment (1:7500 dilution; Amersham Biosciences), sheep anti-mouse IgG (γ-H270, fragment (1:7500 dilution; Amersham Biosciences), and peroxidase-conjugated donkey anti-Goat IgG (0.08 μg/ml; Santa Cruz Biotechnology, Inc.) were used as secondary antibodies.

Pulse-chasing labeling using [35S]methionine/cysteine and subsequent immunoprecipitation experiments were performed as described previously (17). Sucrose gradient fractionation was performed as described previously (19), except that the cells were converted to spheroplasts in the presence of 2 mM CaCl2, which stabilizes spheroplasts.

LCB Kinase Assays—LCB kinase assays were performed using [γ-32P]ATP and D-erythro-sphingosine (Maturea LLC, Pleasant Gap, PA) as substrates. Details were as described previously (17). Radioactivity associated with S1P was quantified using a Fuji Photo Film BAS-2500 bioimaging analyzer.

Quantification of LCBS and LCBPs—Lipid extraction and LCB/LCBP separation were performed by a method previously applied to sphingosine/SIP (21). We confirmed that the unlabeled LCBS and LCBPs were effectively extracted and separated by this method. Cells (1 A600 nm unit) grown in YPD medium at 30 °C were suspended in 100 μl of water, and lipids were extracted by adding 600 μl of 1:2 (v/v) chloroform/methanol, 16 μl of 0.1 M NH4OH, 400 μl of chloroform, and 400 μl of 1 M KCl. After vigorous mixing, the phases were separated by centrifugation, and the organic phase containing LCBs and the aqueous phase containing LCBPs were collected. To quantify the amount of LCBs, the dried organic phase was first suspended in 2 μl of dimethyl sulfoxide, and then 187 μl of buffer containing 20 mM Tris-HCl (pH 8.0) and 0.25% Triton X-100 was added. The samples were subjected to the LCB kinase assay described above using purified Lcb4p (10 ng) as the enzyme.

For quantification, LCBPs were first converted to LCBs by alkaline phosphatase and processed as described above. The aqueous phase was mixed with 95 μl of buffer containing 500 mM Tris-HCl (pH 8.0) and 10 mM MgCl2 and with 10 units of calf intestine alkaline phosphatase (Roche Diagnostics). After a 1-h incubation at 37 °C, the reaction was terminated by adding 32 μl of HCl and 600 μl of chloroform. After vigorous mixing, the phases were separated by centrifugation, and the organic phase was collected and dried.

RESULTS

Isolation of PHS-resistant Yeast Mutants—Exogenous PHS is known to cause growth arrest in yeast (22). Because PHS induces down-regulation of amino-acid permeases, auxotrophic cells are more sensitive to PHS compared with autotrophic cells (22). SEY6210 cells, which carry the leu2 trpl mutation and so are tryptophan and leucine auxotrophs, were found to be sensitive to 12.5 μM PHS. In contrast, SEY6210 cells harboring the pRS314 (TRPI1) and pRS315 (LEU12) plasmids were resistant to as much as 15 μM PHS (TABLE TWO).

Exogenous PHS is readily imported into yeast cells and converted to PHS1P by Lcb4p. In turn, PHS1P is degraded by Dpl1p to a long-chain fatty aldehyde and phosphoethanolamine or by Lcb3p to PHS. Because overaccumulated PHS1P has toxic effects (23–25), the toxicity of exogenously added PHS is determined not only by the PHS itself, but also by PHS1P. In growth studies, the Δdpl1 cells (KHY13/pRS315) were more sensitive to PHS compared with the wild-type cells (SEY6210/pRS314/pRS315), whereas the Δdpl1 Δlcb4 double mutant cells (KHY22) were highly resistant (TABLE TWO). These results indicate that the effect of PHS1P is more prominent than that of PHS in cells autotrophic for tryptophan and leucine.

A Δlcb3 or Δdpl1 single mutation does not affect cell growth, but a double deletion mutation is lethal or impairs growth severely depending on the yeast background (9, 24, 25). In the yeast background used here (SEY6210), the Δdpl1 Δlcb3 cells (KHY388) were able to grow, but did so poorly (TABLE TWO). Moreover, when grown on YPD plates, their colony sizes were highly heterogeneous. When KHY388 (Δdpl1 Δlcb3) cells were visualized by 4,6-diamidino-2-phenylindole staining under an immunofluorescence microscope, most cells contained nuclei with aberrant morphology (Fig. 1, right panels). Some had fragmented or multiple nuclei, and even cells with no nucleus were observed. This morphology was quite different from that observed in the wild-type cells, which each possessed a single round nucleus (Fig. 1, left panels). The heterogeneous growth of the cells might be the result of differences in the extent of the chromosomal integrity.

We isolated KHY389 cells from KHY388 (Δdpl1 Δlcb3) cells as clones exhibiting homologous growth. Although KHY389 cells still grew more slowly than the wild-type cells (TABLE TWO), the morphology of their nuclei was indistinguishable from that of the wild-type cells (data not shown). The introduction of plasmids encoding LCB3 or DPL1 into KHY389 cells only slightly suppressed their slow growth (data not shown). These results suggest that KHY389 cells carry a suppressor mutation that is a disadvantage for growth but that confers tolerance to overaccumulated LCBPs. However, KHY389 cells still retained high sensitivity to exogenous PHS because they could not grow on YPD plates containing 7.5 μM PHS (TABLE TWO). These results indicate that KHY389 cells are less responsive to LCBPs compared with the original KHY388 (Δdpl1 Δlcb3) cells, but are still more sensitive compared with the wild-type or Δdpl1 cells.
In our previous study (19), we introduced transposon insertion mutations into KHY13 (Δdpl1) cells and screened for mutants that were resistant to 15 μM PHS. In addition to the expected LCB4 gene, we identified PDR5 and DHH1 as genes conferring PHS resistance (19). To discover additional genes, we repeated the screening using transposon-inserted KHY389 cells and a lower concentration of PHS (7.5 μM). We expected low concentrations of PHS to provide an advantage in isolating genes involved in LCBP signaling and in regulation of LCBP synthesis rather than those involved in LCB signaling. We obtained transposon-inserted mutants that exhibited PHS resistance at a frequency of ~1/600 mutants. Of these, 36 mutants were randomly selected, and their sites of transposon insertion were determined. We identified eight genes (PBP1, HEM14, UFD4, HMG1, TPS1, KES1, WHI2, and ERG5) that were involved in PHS resistance, in addition to the previously obtained LCB4 and PDR5 genes (TABLE THREE). Interestingly, these eight genes, four (HMG1, ERG5, HEM14, and KES1) are known to be involved in ergosterol synthesis or distribution, either directly or indirectly.

**PHS Sensitivity in the Mutants of Identified Genes**—We next investigated the PHS sensitivity of each isolated yeast mutant. Because all the transposon-inserted mutants contain the LEU2* marker within the transposon, we created control KHY406 cells by introducing the LEU2* gene into KHY389 cells, thereby providing uniform auxotrophic conditions. As shown in TABLE FOUR, all the isolated PHS-resistant yeast mutants were able to grow in the presence of 7.5 μM PHS, in contrast to control KHY406 cells, although the degree of PHS sensitivity varied. The lcb4 mutants exhibited the strongest PHS resistance, although the hmg1, pdr5, and ufd4 mutants were also quite resistant. In contrast, the whi2 and hem14 mutants exhibited only modest PHS resistance. The growth of the hem14 mutants was slower than that of control KHY406 cells in the absence of PHS and was inhibited slightly further by 7.5 μM PHS. The PHS sensitivity of the tps1 mutants differed completely from that of the other mutants. Their growth in the absence of PHS was extremely slow, yet PHS did not inhibit growth further, but rather promoted it, so that in the presence of 10 μM PHS, the tps1 mutants grew as slowly as the whi2 mutants. The pph1, kes1, and erg5 mutants displayed the weakest resistance to PHS. Additionally, the PHS sensitivities of all these transposon-inserted mutants were identical to those of their respective deletion mutants, which we constructed using KHY406 (Δdpl1 Δlcb3 sup) cells as the parental strain (data not shown). These results confirm that the genes identified here by transposon mutagenesis are indeed responsible for PHS resistance.

The parental strain used for transposon mutagenesis (KHY389) may carry an unidentified suppressor mutation as described above. To exclude any indirect effects of such a suppressor, we introduced the deletion mutation of each PHS-resistant gene into the suppressor-less Δdpl1 single mutation-containing KHY360 cells and examined their PHS sensitivities. The growth of KHY360 (Δdpl1) cells was inhibited by PHS in a dose-dependent manner, so that at 12.5 μM PHS, the cells grew only weakly (TABLE FIVE). Most of the mutations introduced here conferred apparent PHS resistance, and the order of the strength of this resistance was similar to that observed in the transposon-inserted mutants presented in TABLE FOUR. However, the PHS resistance of the Δhem14 and Δerg5 cells was rather unclear (TABLE FIVE). The growth rate of the Δhem14 cells was similar to that of control KHY360 cells at 10 μM PHS and even weaker at 12.5 μM PHS. However, considering that the growth of the Δhem14 cells was slow in the absence of PHS and that 10 μM PHS did not inhibit this already slow growth further, we concluded that the Δhem14 mutation did confer PHS resistance. On the other hand, the Δerg5 cells exhibited PHS sensitivity similar to that of control KHY360 cells at all concentrations of PHS tested. The erg5 mutation also exhibited the weakest PHS resistance in the KHY389 (Δdpl1 Δlcb3 sup) background (TABLE FOUR), suggesting that the effect of this mutation was not detectable in the KHY360 (Δdpl1) background.

**Accumulation of LCBs in PHS-resistant Yeast Mutants**—Reduced accumulation of exogenous PHS caused by a decrease in its uptake or an

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**TABLE THREE**

| Genes identified in this study and their known functions |
|------------------------------------------------------|
| Gene | No. | Function |
|------|-----|----------|
| LCB4 | 11  | LCB kinase |
| PBP1 | 5   | Poly(A)-binding protein-binding protein |
| HEM14| 4   | Heme biosynthesis (protoporphyrinogen oxidase) |
| UFD4 | 4   | Ubiquitin-protein isopeptide ligase |
| HMG1 | 3   | Ergosterol biosynthesis (hydroxymethylglutaryl-CoA reductase) |
| TPS1 | 3   | α,β-Trehalose-phosphate synthase |
| PDR5 | 2   | Xenobiotically-transporting ATPase |
| KES1 | 2   | Oxysterol-binding protein homolog |
| WHI2 | 1   | Regulator of stress response element-mediated gene expression |
| ERG5 | 1   | Ergosterol biosynthesis (C22 sterol desaturase) |
| Total| 36  |          |

*Number of transposon-inserted mutants isolated this study.

**TABLE FOUR**

| PHS sensitivity in PHS-resistant yeast mutants |
|-----------------------------------------------|
| The isolated transposon-inserted mutants and the control cells (Δdpl1 Δlcb3 sup) were cultured at 30 °C for 2 days on YPD plates containing 7.5 or 10 μM PHS as indicated. The PHS sensitivity was evaluated in seven grades (–, –/–, +, ++, ++++, ++++, +++++) according to colony size. |

| Strain | PHS |
|--------|-----|
| Control (KHY406) | 0 μM | 7.5 μM | 10 μM |
| lcb4::mTn-lacZ::LEU2 | ++++++ | ++++ | +/– |
| pph1::mTn-lacZ::LEU2 | ++++ | ++ | + |
| hem14::mTn-lacZ::LEU2 | +++ | ++ | + |
| ufd4::mTn-lacZ::LEU2 | +++ | +++ | + |
| hmg1::mTn-lacZ::LEU2 | ++++ | ++++ | + |
| tps1::mTn-lacZ::LEU2 | + | ++ | + |
| pdr5::mTn-lacZ::LEU2 | +++ | +++ | + |
| kes1::mTn-lacZ::LEU2 | ++++ | ++ | – |
| whi2::mTn-lacZ::LEU2 | ++++ | +++ | + |
| erg5::mTn-lacZ::LEU2 | +++ | +++ | – |

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TABLE FIVE

PHS sensitivity in the constructed deletion mutants

| Strain       | PHS   | 0 μM | 10 μM | 12.5 μM |
|--------------|-------|------|-------|---------|
| Control (KHY360) | +++   | +    | +     | +/--    |
| Δcb4        | ++++  | ++++ | ++++  | +/--    |
| Δpbl1       | ++++  | ++   | ++    | ++/++   |
| Δhem14      | ++    | +    | +     | +/++    |
| Δufd4       | ++++  | ++++ | ++    | ++/++   |
| Δhmg1       | ++++  | ++   | ++    | ++/++   |
| Δtps1       | ++/—  | +    | +     | +       |
| Δpdr5       | ++++  | ++   | ++    | ++/++   |
| Δkes1       | ++++  | ++   | ++    | ++/++   |
| Δwhi2       | ++++  | ++   | ++    | ++/++   |
| Δerg5       | ++++  | ++   | +     | +/++    |

increase in its efflux is considered to be one mechanism by which mutants acquire PHS resistance. To investigate this possible mechanism in the transposon-inserted mutants, the cells were incubated with [3H]DHS for 1 h at 30°C, and the amounts of accumulated [3H]DHS were measured. [3H]DHS accumulation in the pdr5 mutants was reduced by ~25% compared with that in the control cells (Fig. 2). This was similar to our previous results for pdr5 deletion mutants, which exhibit an enhanced efflux of LCBs attributable to an increase in the LCB-specific translocate/transporter Rsb1p (19). In contrast, the accumulation of [3H]DHS was markedly increased in the hem14 and hmg1 mutants. Other mutations did not affect the accumulation of [3H]DHS significantly, although slight increases were observed in the pbl1, kes1, and erg5 mutants. These results suggest that the PHS resistance of the mutants, other than the pdr5 mutants, is not caused by a reduction in [3H]DHS accumulation.

Reduced Levels of Lcb4p in the Ergosterol-related Mutants—Decreased production of PHS1P from imported PHS is another possible mechanism for acquiring PHS resistance. Therefore, we next examined whether any of the PHS-resistant mutations affected the protein levels of the major LCB kinase Lcb4p. Cell lysates were prepared from each deletion mutant in the KHY360 (∆dpl1) background and subjected to immunoblotting with anti-Lcb4p antibodies. Two protein bands corresponding to phosphorylated and non-phosphorylated forms of Lcb4p (17) were detected in the control cells, and the upper, phosphorylated band was predominant (~70% of total Lcb4p) (Fig. 3, A and B). The Δpbl1, Δufd4, Δtps1, Δpdr5, and Δwhi2 cells had equivalent levels of Lcb4p with similar phosphorylation profiles. On the other hand, the amount and/or phosphorylation of Lcb4p was reduced in the ergosterol-related mutants, i.e., the Δhem14, Δhmg1, Δkes1, and Δerg5 cells (Fig. 3, A and B). In the Δhem14 cells, the amount of Lcb4p was greatly reduced to ~20% of the level found in the control cells, and the phosphorylated form of Lcb4p was scarcely detected. In the Δhmg1 cells, the total amount of Lcb4p (phosphorylated and non-phosphorylated) was slightly decreased to ~80% of the total in the control cells, with the phosphorylated form declining to ~50%. In the Δkes1 and Δerg5 cells, the total amounts of Lcb4p were similar to the levels observed in the control cells; however, a decrease in the phosphorylated form was observed (Fig. 3, A and B). In particular, the non-phosphorylated form was more prevalent than the phosphorylated form in the Δkes1 cells, with a slight but reproducible increase apparent in the non-phosphorylated form in the Δerg5 cells (Fig. 3, A and B; see also Fig. 4B).

We also examined the in vitro LKB kinase activity of the ergosterol-related mutants. As shown in Fig. 3C, the LKB kinase activities in the Δhem14 and Δhmg1 cell lysates were reduced to ~30 and 85%, respectively, of the activity observed in the control cells. In contrast, the LKB kinase activities in the Δkes1 and Δerg5 cells were similar to the activity in the control cells. These results suggest that the PHS resistance of the ergosterol-related mutants is caused by changes in the expression and/or phosphorylation of Lcb4p.

Effect of Sterol Composition on the Phosphorylation of Lcb4p—Because mutations in the ergosterol-related genes (HEM14, HMG1, KES1, and ERG5) resulted in a reduction in the phosphorylation of Lcb4p, we considered that the sterol composition might influence the phosphorylation of this protein. Genes involved in the early stages of ergosterol synthesis are essential for cell growth; however, genes involved in the later stages of synthesis (ERG6, ERG2, ERG3, ERG5, and ERG4) (Fig. 4A) can be deleted without interrupting cell growth, and disruption of any is known to cause changes in the sterol composition (26, 27). Using the Δerg6, Δerg2, Δerg3, and Δerg5 cells in the KHY360 (∆dpl1) background, we investigated the roles of ergosterol in the phosphorylation of Lcb4p by immunoblotting. The phosphorylation of Lcb4p was reduced in all the ergosterol mutants, although the degree of reduction varied (Fig. 4B). The Δerg6 cells exhibited the greatest reduction in phosphorylation, and the Δerg3 and Δerg2 mutations also significantly affected the phosphorylation of Lcb4p. Although a single deletion of the ERG2, ERG3, or ERG5 gene had only a moderate or weak effect on the phosphorylation of Lcb4p, double deletions induced striking changes in phosphorylation (Fig. 4B). These results indicate that changes in the sterol composition can indeed cause a reduction in the phosphorylation of Lcb4p. On the other hand, the levels of Lcb4p appear not to be affected.

Next, we measured the intracellular amounts of LCBs and LCBPs in the deletion mutants of the PHS-resistant genes and the ergosterol-synthesizing genes, all in the KHY360 (∆dpl1) background (Fig. 5). As
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LCBs were significantly reduced in the Δhem14 cells. Unexpectedly, both LCBs and LCBPs were increased in the ergosterol-synthesizing mutants Δhmgl, Δerg2, Δerg3, Δerg6, Δerg2 Δerg3, and Δerg3 Δerg5. On the other hand, the LCB/LCBP levels were only slightly affected or unchanged in the other mutants.

Involvement of the Heme-dependent Transcription Factor Hap1p in the Expression of Lcb4p—Although changes in the sterol composition seemed not to affect the Lcb4p levels (Fig. 4B), the protein levels were greatly and slightly reduced in the Δhem14 and Δhmgl cells, respectively (Fig. 3A). To investigate whether the reduction was caused by reduced synthesis or increased degradation of the protein, we performed a pulse-chase experiment in the Δhem14 cells. As shown in Fig. 6 (A and B), the synthesis of Lcb4p was lower in the Δhem14 cells than in the wild-type cells by -2-fold. Degradation of Lcb4p in the Δhmgl cells was not accelerated, but instead was delayed, indicating that the reduction in Lcb4p was caused solely by decreased synthesis.

Hem14p is a protoporphyrogen oxidase that catalyzes the late step of heme synthesis (28, 29). Hmg1p is involved in the synthesis of farnesyl diphosphate (30), which is also required for synthesis of certain hemes (hemes O and A) (31). Some genes involved in ergosterol biosynthesis, including HMG1 and ERG11, are regulated by heme at the transcriptional level (32, 33), and the transcription factor Hap1p is known to be involved in this regulation (34).

We investigated whether Hap1p might be involved in the transcriptional regulation of Lcb4p by examining Lcb4p expression in Δhap1 cells. Lcb4p levels were reduced in the Δhap1 cells to about half those found in the wild-type cells (Fig. 6C), not as drastic a reduction as in the Δhem14 cells. Furthermore, the amount of phosphorylated Lcb4p was also reduced in the Δhap1 cells. This reduction may be an indirect result of reduced transcription of the ergosterol-synthesizing genes HMG1 and ERG11. These data suggest that defects in heme synthesis in the Δhem14 and Δhmgl cells affect the activity of Hap1p, resulting in a reduction in the expression of Lcb4p.

Effect of the erg Mutations on the Localization of Lcb4p—The cause of the reduced phosphorylation of Lcb4p in the erg mutants was not clear. One possible mechanism would be the altered cellular localization of Lcb4p in these mutants. Thus, we performed a sucrose gradient fractionation. In the wild-type cells, Lcb4p exhibited two peaks: a major peak in fractions 3–5 and a minor peak in fraction 10 (Fig. 7A). The major peak may represent endoplasmic reticulum (ER)-resident Lcb4p because the ER membrane protein Dpm1p was also highest in these fractions. The minor peak coincided with the plasma membrane marker Pma1p. These results suggest that Lcb4p is localized mainly in the ER, but that a small amount of Lcb4p also resides in the plasma membrane.

We also examined fractions from the Δerg6 cells, which exhibited a great reduction in the phosphorylation of Lcb4p (Fig. 4B). In these cells, plasma membrane-localized Lcb4p was hardly detected, and most of Lcb4p was localized in the ER fractions (Fig. 7B). These results indicate that altered ergosterol composition prevents Lcb4p from localizing in the plasma membrane.

DISCUSSION

In this study, we screened for PHS-resistant genes in yeast using transposon mutagenesis. In addition to the previously identified LCB4 and PDR5 genes, we identified eight other genes (PBPI, HEM14, UFD4, HMG1, TPS1, KES1, WH12, and ERG5) that appear to be involved in PHS resistance (TABLE THREE). Of these eight genes, four (HMG1, ERG5, HEM14, and KES1) were identified as ergosterol-related genes, and two of these (HMG1 and ERG5) are directly involved in ergosterol synthesis. Hmg1p and its homolog Hmg2p redundantly catalyze the

FIGURE 3. The amount and/or phosphorylation of Lcb4p is reduced in Δhem14, Δhmgl, Δkes1, and Δerg5 cells. A and B, total proteins (10 μg) prepared from TS14 (Δlc4), KHY479 (Δpdp1), FKY47 (Δhem14), KHY481 (Δwdr4), TS33 (Δhmgl), TS4 (Δpdp1), KHY364 (Δpdr3), TS45 (Δkes1), KHY480 (Δwdr2), FKY48 (Δerg5), and KHY360 (wild-type) cells were separated by SDS-PAGE and transferred to membranes for immunoblotting. Proteins were detected with anti-Lcb4p antibodies or, to demonstrate uniform protein loading, with anti-Pk1p antibodies (A). The intensity of phosphorylated (p) and nonphosphorylated Lcb4p was quantified using NIH Image Version 1.62 software (B). Each value is presented as a percentage of the amount of phosphorylated Lcb4p relative to that of total Lcb4p and represents the mean ± S.D. from three independent experiments. Statistically significant differences between values for the wild-type and mutant cells are indicated, *, p < 0.001. C, cell lysates were prepared from FKY47 (Δhem14), TS33 (Δhmgl), TS45 (Δkes1), FKY48 (Δerg5), and KHY360 (wild-type) cells. Samples (20 μg of protein) were subjected to an in vitro LCB kinase activity assay using a mixture of [γ-32P]ATP, 1 μM unlabeled ATP, and 50 μM sphingosine and incubated at 37 °C for 15 min. Lipids were extracted and separated by TLC. Radioactivity associated with the resulting S1P was quantified using a Fuji Photo Film BAS-2500 bioimaging analyzer. Each value is presented as a percentage relative to the LCB kinase activity associated with the control cells and represents the mean ± S.D. from three independent experiments. Statistically significant differences between values for the wild-type and mutant cells are indicated, *, p < 0.001; **, p < 0.01.
rate-limiting step, the conversion of hydroxymethylglutaryl-CoA to mevalonic acid, and \( hmg1 \) cells exhibit largely reduced enzyme activity (35). Erg5p is the enzyme that desaturates C-22 of the side chain in the late step of ergosterol synthesis (36). Hem14p converts protoporphyrinogen IX to protoporphyrin IX in the late step of heme synthesis (28, 29), so in \( hem14 \) cells, the function of heme-containing proteins such as the ergosterol synthesis-related enzymes Erg5p and Erg11p (36, 37) would be reduced. Moreover, the expression of \( HMG1 \) and \( ERG11 \), regulated by heme at the transcriptional level (32, 33), would be also decreased. Finally, Kes1p, an oxysterol-binding protein homolog, is thought to be involved in ergosterol transport from the Golgi to the plasma membrane, and although total ergosterol levels in \( kes1 \) cells are unchanged, the levels on the cell surface are reduced (38).

One possible mechanism responsible for the PHS resistance of these mutants would be a reduction in LCB accumulation; however, DHS accumulation was decreased only in the \( pdr5 \) mutants (Fig. 2). We recently demonstrated that the LCB-releasing activity of \( pdr5 \) mutants is increased due to up-regulation of the LCB-specific translocase/transporter Rsb1p (19). In contrast, accumulation of [\(^{3}H\)]DHS was somewhat increased in the \( hem14 \) and \( hmg1 \) mutants (Fig. 2). Either mutation (\( hem14 \) or \( hmg1 \)) affects ergosterol synthesis, so increased accumulation would be consistent with a previous finding that the uptake of certain compounds is increased in ergosterol mutants because of enhanced membrane permeability (39).

The \( hem14 \) and \( hmg1 \) cells, which are defective in both ergosterol and heme synthesis, had decreased Lcb4p levels (Fig. 3A). This decrease was especially significant in the \( hem14 \) cells, and intracellular LCBPs were reduced to \( \sim 25 \% \) of the levels in the wild-type cells (Fig. 5B); such a loss may cause the \( hem14 \) cells to be resistant to exogenous PHS. The
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Δhem14 and Δhmg1 cells also exhibited reduced phosphorylation of Lcb4p (Fig. 3, A and B), yet cells carrying mutations specifically affecting ergosterol synthesis (Δerg6, Δerg2, Δerg3, and Δerg5) exhibited only reduced phosphorylation (Fig. 4B). We found that Lcb4p expression was partially regulated by the heme-dependent transcription factor Hap1p (Fig. 6C). Therefore, it is likely that the reduction in the Lcb4p levels in the hem14 and hmg1 mutants is caused by an impairment in heme synthesis and not by one in ergosterol synthesis. However, the amount of Lcb4p in the Δhem14 cells was still lower than in the Δhap1 cells (Fig. 6C). One possible explanation for this is that other transcription factors affected by the Δhem14 mutation are also involved in the expression of Lcb4p. Alternatively, the decreased expression of Lcb4p in the Δhem14 cells might be due solely to the reduced activity of Hap1p. However, the difference in the amount of Lcb4p between the Δhap1 and Δhem14 cells might be caused by a difference in their growth rates because the growth rate of the Δhem14 cells was significantly slow (TABLES FOUR AND FIVE).

In our sucrose density gradient experiment (Fig. 7) and an immunofluorescence microscopic analysis of the wild-type cells, Lcb4p was found to be localized mainly in the ER, with some presence in the plasma membrane, although this pattern differs from that found in other studies using a C-terminal hemagglutinin tag, which may have interfered with localization (40, 41). In the mutant screening, PHS was added exogenously, so perhaps plasma membrane-localized Lcb4p is important for conversion to PHS1P of only imported PHS (i.e. that which is localized in the plasma membrane). This would appear to be the reason that the hmg1 and erg5 mutants were isolated by our screening, although they can normally produce LCBPs from endogenous LCBs (Fig. 5), which are localized mainly in the ER. We also speculate that Lcb4p is phosphorylated at the plasma membrane and that, in the erg mutants, reduced localization at the plasma membrane results in decreased phosphorylation. Additionally, we recently reported that Lcb4p is anchored to the membranes through palmitoylation (42). Because palmitoylation often facilitates recruitment into sterol/sphingolipid-rich microdomains (43, 44), it is possible that the palmitic acid moiety of Lcb4p interacts with ergosterol, which is abundant in the plasma membrane but not in the ER and that this interaction causes the retention of Lcb4p in the membrane.

Interestingly, LCB/LCBP levels were increased in the erg mutants (Fig. 5). Previous studies have shown that ergosterol levels affect the amounts of certain sphingolipid species (45). Moreover, Δarv1 cells, in which intracellular sterol distribution is altered, also harbor defects in sphingolipid synthesis (46). Thus, there seems to be unknown mechanisms that regulate sphingolipid synthesis in response to cellular ergosterol status.

The mutations of the TPS1, WHI2, PBP1, and UFD4 genes affected neither LCB accumulation nor Lcb4p expression/phosphorylation (Figs. 3 and 4). Intracellular LCBPs were also nearly unchanged in these mutants, except for the tsp1 mutant, which exhibited reduced LCB/LCBP levels (Fig. 5), probably because of an indirect growth effect. Nevertheless, it is possible that these genes are involved in LCBP signaling. LCBPs are known to be involved in the heat stress response, and Tps1p and Whi2p indeed also function in this process (47, 48). Future studies are required to reveal the link between these genes and LCBP signals.

Acknowledgment—We thank Dr. Michael Snyder for kindly providing the mTn-lacZ/LEU2-mutagenized yeast genomic library.

REFERENCES

1. Zanolari, B., Friant, S., Funato, K., Sütterlin, C., Stevenson, B. J., and Riezman, H. (2000) EMBO J. 19, 2824–2833

3 T. Sano, A. Kihara, S. Iwaki, and Y. Igarashi, unpublished data.
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