Molecular Biology of the Cell
Vol. 15, 4949–4959, November 2004

Cross Talk between Sphingolipids and Glycerophospholipids in the Establishment of Plasma Membrane Asymmetry

Akio Kihara and Yasuyuki Igarashi*

Department of Biomembrane and Biofunctional Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

Submitted June 8, 2004; Accepted August 24, 2004
Monitoring Editor: Howard Riezman

Glycerophospholipids and sphingolipids are distributed asymmetrically between the two leaflets of the lipid bilayer. Recent studies revealed that certain P-type ATPases and ATP-binding cassette (ABC) transporters are involved in the inward movement (flip) and outward movement (flop) of glycerophospholipids, respectively. In this study of phytosphingosine (PHS)-resistant yeast mutants, we isolated mutants for *PDR5*, an ABC transporter involved in drug efflux as well as in the flop of phosphatidylethanolamine. The *pdr5* mutants exhibited an increase in the efflux of sphingoid long-chain bases (LCBs). Genetic analysis revealed that the PHS-resistant phenotypes exhibited by the *pdr5* mutants were dependent on Rsb1p, a putative LCB-specific transporter/translocase. We found that the expression of Rsb1p was increased in the *pdr5* mutants. We also demonstrated that expression of *RSB1* is under the control of the transcriptional factor Pdr1p. Expression of Rsb1p also was enhanced in mutants for the genes involved in the flip of glycerophospholipids, including *ROS3*, *DNF1*, and *DNF2*. These results suggest that altered glycerophospholipid asymmetry induces the expression of Rsb1p. Conversely, overexpression of Rsb1p resulted in increased flip and decreased flop of fluorescence-labeled glycerophospholipids. Thus, there seems to be cross talk between sphingolipids and glycerophospholipids in maintaining the functional lipid asymmetry of the plasma membrane.

INTRODUCTION

Lipid molecules are not equally distributed between the inner and outer leaflets of the lipid bilayer forming the plasma membrane. For example, phosphatidylcholine (PC) is abundant in the outer leaflet, whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly found in the inner leaflet (Schroit and Zwaal, 1991). The trans-bilayer movement of phospholipids in a membrane model is very slow (Kornberg and McConnell, 1971) in contrast to such movement through biogenic membranes. Thus, the existence of enzymes that catalyze the translocation of phospholipids has long been suspected. Recent studies have demonstrated that three major groups of enzymes, P-type ATPases, ATP-binding cassette (ABC) transporters, and scramblases, are active in the trans-bilayer movement of glycerophospholipids (Bevers *et al*., 1999; Williamson and Schlegel, 2002).

One particular subfamily of the P-type ATPases, the aminophospholipid translocases, are involved in the inward movement (flip) of PS and PE. Mammalian ATPase II, the product of the class 1a gene, was the first aminophospholipid translocase identified (Tang *et al*., 1996). The yeast *Saccharomyces cerevisiae* contains five such translocases: Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p. Deletion of the *DRS2* gene reportedly resulted in a loss in the uptake of labeled PS (Tang *et al*., 1996), although contrary results have been presented by other groups (Siegmund *et al*., 1998; Marx *et al*., 1999). Recently, Dnf1p and Dnf2p were found to be involved in glycerophospholipid translocation in the plasma membrane (Pomorski *et al*., 2003). That report demonstrated that Drs2p is not localized in the plasma membrane but rather is found in the Golgi complex where it functions in the flip of glycerophospholipids (Pomorski *et al*., 2003). Dnf3p, another P-type ATPase closely related to the three proteins mentioned above, seems to have overlapping functions. Mutants carrying the quadruple deletion Δ*drs2* Δ*dnf1* Δ*dnf2* Δ*dnf3* were inviable; however, triple mutants (Δ*drs2* Δ*dnf1* Δ*dnf2*, Δ*drs2* Δ*dnf2* Δ*dnf3*, Δ*drs2* Δ*dnf1* Δ*dnf3*, and Δ*dnf1* Δ*dnf2* Δ*dnf3*) survived (Hua *et al*., 2002). Single Δ*drs2* mutants exhibited late Golgi defects such as decreased processing of pro-α-factor and an accumulation of abnormal Golgi cisternae (Chen *et al*., 1999). Additionally, transport of alkaline phosphatase to the vacuole was defective in the Δ*drs2* Δ*dnf1* cells (Hua *et al*., 2002). Moreover, the Δ*dnf1* Δ*dnf2* Δ*dnf3* mutation caused a mislocalization of GFP-tagged Snc1p, suggesting the involvement of Dnf1p, Dnf2p, and Dnf3p in the endosome-to-*trans*-Golgi network transport (Hua *et al*., 2002). Finally, a defect in endocytosis was observed in the Δ*dnf1* Δ*dnf2* Δ*drs2* mutants (Pomorski *et al*., 2003). These data suggest that proper glycerophospholipid asymmetry is important in the maintenance of organelle structure and intracellular trafficking.

An additional gene, *ROS3*/*LEM3*, was identified in separate studies screening mutants hypersensitive to a tetracyclic peptide that binds specifically to PE (Kato *et al*., 2002) or resistant to alkylphosphocholine drugs (Hanson *et al*., 2003). *ROS3* is required for glycerophospholipid translocation across the plasma membrane (Kato *et al*., 2002; Hanson *et al*., 2003). Recently, Ros3p and its homolog Cdc50p were shown

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04–06–0458. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–06–0458.

* Corresponding author. E-mail address: yigarash@pharm.hokudai.ac.jp.

© 2004 by The American Society for Cell Biology

to interact with Dnf1p and Drs2p, respectively, and to function in the establishment of cell polarity (Saito *et al*., 2004).

Certain ABC transporter family members are involved in the outward movement (flop) of glycerophospholipids. For instance, the transporter human MDR1 acts as a lipid translocase with broad specificity, whereas mouse mdr2 and human MDR3 function specifically in the translocation of PC (Smit *et al*., 1993; Smith *et al*., 1994; van Helvoort *et al*., 1996). More recently, involvement in the outward transport of labeled PS was demonstrated for the ABC transporter MRP1 by using red blood cells (Dekkers *et al*., 1998). In yeast, Pdr5p and Yor1p, also of the ABC transporter family, have been shown to be involved in the flop of PE (Decottignies *et al*., 1998).

In addition to glycerophospholipids, sphingolipids are major components of the eukaryotic plasma membrane. Mammalian sphingolipids include sphingomyelin and hundreds of glycosphingolipids, whereas in yeast such as *S. cerevisiae* only three sphingolipids exist, all containing *myo*-inositol. Ceramide, the backbone of sphingolipids, is composed of a long-chain base (LCB) and a fatty acid linked by an amide bond. In mammalian cells the major LCB is sphingosine. Again, sphingosine does not exist in *S. cerevisiae*; instead, dihydrosphingosine (DHS) and phytosphingosine (PHS) serve as LCBs. In mammalian cells, sphingomyelin metabolites such as ceramide, sphingosine, and sphingosine 1-phosphate act as bioactive lipid molecules to regulate cell growth, differentiation, motility, and apoptosis (Hannun *et al*., 2001; Spiegel and Milstien, 2003). In yeast, the two LCBs and their phosphorylated forms, the long-chain base 1-phosphates (LCBPs), also function as signaling molecules involved in heat stress response, endocytosis, cell cycle arrest, Ca^{2+} mobilization, and diauxic shift (Obeid *et al*., 2002).

Sphingomyelin and glycosphingolipids are known to be localized in the outer leaflet of the plasma membrane, whereas the distribution of ceramide and LCBs between the two leaflets has not been determined. Previous biochemical analysis by using mouse liver suggested that sphingolipid synthesis is initiated and also proceeds on the cytoplasmic side of the endoplasmic reticulum (ER) membrane, to the point of dihydroceramide synthesis (Mandon *et al*., 1992). LCBs seem to be located on both sides of the ER membrane. LCBs are converted to LCBPs by LCB kinases localized in the cytoplasm and can associate peripherally with the membrane on the cytoplasmic side. However, the LCBP phosphatase Lcb3p also generates LCBs from LCBP in the ER lumen (Kihara *et al*., 2003). Because galactosylceramide and glucosylceramide are synthesized on the luminal side of the ER and on the cytoplasmic side of the Golgi apparatus, respectively (Coste *et al*., 1986; Futerman and Pagano, 1991; Jeckel *et al*., 1992; Sprong *et al*., 1998), their precursor, ceramide, also may exist in both sides. Once synthesized, glucosylceramide on the cytoplasmic side is translocated to the luminal side of the Golgi apparatus, where conversion to lactosylceramide and complex glycosphingolipids takes place (Lannert *et al*., 1994). Sphingomyelin is also synthesized in the luminal side of the Golgi apparatus (Futerman *et al*., 1990; Jeckel *et al*., 1992).

Although the mechanism for glycerophospholipid translocation has been well studied, a similar system for sphingolipid translocation has yet to be disclosed. Recently, Rsb1p was identified as a putative LCB transporter/translocase (Kihara and Igarashi, 2002). Overexpression of Rsb1p resulted in an increased efflux of LCBs, whereas disruption of its gene greatly reduced such efflux (Kihara and Igarashi, 2002). Here, we reveal that Rsb1p expression is enhanced by the deletion of the ABC transporter genes *PDR5* and *YOR1*. Moreover, the expression was up-regulated in mutants defective in the inward translocation of glycerophospholipids. These results suggest that altered membrane asymmetry can trigger the expression of *RSB1*.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Media

S. cerevisiae strains used are listed in Table 1. The Δ*dpl1::TRP1* (Kihara and Igarashi, 2002), Δ*rsb1::HIS3* (Kihara and Igarashi, 2002), and Δ*lcb4::LEU2* (Kihara *et al*., 2003) constructions were described previously. The Δ*pdr5::KanMX4*, Δ*dhh1::KanMX4*, Δ*pdr1::KanMX4*, Δ*yor1::KanMX4*, Δ*ros3::KanMX4*, Δ*dnf1::KanMX4*, Δ*dnf2::KanMX4*, Δ*pdr3::KanMX4*, Δ*ros3::HIS3*, and Δ*yor1::HIS3* cells were constructed by replacing their entire open reading frames with the *KanMX4* marker or the *HIS3* marker. For construction of the Δ*pdr5::URA3* and Δ*dnf1::HIS3* cells, the 2.0-kb *Bam*HI-*Pme*I region in the *PDR5* gene and the 0.5-kb *Sma*I-*Nru*I region in the *DNF1* gene were replaced with the respective auxotrophic markers. The cells were grown either in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or in synthetic complete (SC) medium (0.67% yeast nitrogen base and 2% glucose) containing nutritional supplements.

The pAK80 plasmid is a yeast cloning vector for *TDH3* promoter-dependent expression (Kihara and Igarashi, 2002). The pAK90 and pAK170 plasmids are derivatives of pAK80 and encode *RSB1* and *3xHA-RSB1*, respectively (Kihara and Igarashi, 2002).

Construction of KHY426 (*rsb1::3xHA-RSB1*) cells was as follows. KHY403 (SEY6210, Δ*rsb1::URA3*) cells were first created by replacing the 0.6-kb *Eco*RV-*Eco*RV region in the *RSB1* gene with the *URA3* marker. The pAK464 plasmid, in which the 5′-upstream region (−317 to −1) of the *RSB1* gene was connected to the *3xHA-RSB1* construct derived from the pAK170 plasmid, and the primers 5′-CCAGAATCAATAGAAATAAGAAGAG-3′ and 5′-TATGGAGG-TAAACTAACGCTCCTGC-3′ were used for amplification of the 5′-upstream-*3xHA-RSB1* region by polymerase chain reaction (PCR). Thus, generated fragments were then introduced into the KHY403 cells, and cells that lost the *URA3* marker were selected with 50 μg/ml 5-fluoro-orotic acid. Genomic DNAs were prepared from some of the obtained colonies, and replacement of Δ*rsb1::URA3* with *3xHA-RSB1* was examined by PCR. One of the clones, KHY426, exhibited the *rsb1::3xHA-RSB1* genotype.

To identify the 5′-upstream region of the *RSB1* gene required for the expression of Rsb1p, pAK556, pAK549, and pAK550 were created as follows. Each 5′-upstream-*3xHA-RSB1* region was amplified by PCR by using genomic DNA prepared from KHY426 as a template, a common primer (5′-GTA-CATATTACGATGTCGAAATATAAGG-3′), and respective primers (pAK556, 5′-TTAGAGCGCGTGTTGAAATATAGTC-3′; pAK549, 5′-AGCAT-TCTTGCTCCGTCATATTTCC; pAK550, 5′-AAGATATGGTCTCCGTCG-GTCCTCTG-3′). The amplified region was then inserted into the yeast expression vector pRS423 (Christianson *et al*., 1992).

Isolation of a PHS-resistant Mutant by Transposon Mutagenesis

A yeast genomic library that had been mutagenized by random insertion of the transposon mTn-*lacZ/LEU2* (Burns *et al*., 1994) was kindly provided by Dr. Michael Snyder (Yale University, New Haven, CT). The genomic library was digested with *Not*I to excise yeast DNA fragments, and the resulting fragments were used for the transformation of KHY13 cells. Transformants were selected by incubating on SC medium lacking leucine. Pooled transformants were then plated on YPD medium containing 15 μM PHS and 0.0015% Nonidet P-40 as a dispersant. After incubating at 30°C for 2 d, we obtained PHS-resistant mutants at a frequency of about 1 to 9000. The sites of insertion were then determined according to the manual of the Yale Genome Analysis Center (http://ygac.med.yale.edu/).

Assaying [^{3}H]DHS Uptake and Release

Yeast cells grown at 30°C to 1 OD_{600} unit/ml were treated with [4,5-^{3}H]DHS (0.5 μCi/1 OD_{600} cells) (50 Ci/mmol) (American Radiolabeled Chemicals, St. Louis, MO), which had been complexed with 1 mg/ml fatty acid-free bovine serum albumin (BSA) (A-6003; Sigma-Aldrich, St. Louis, MO), and incubated for various time periods. At each time point, cells equivalent to 0.45 OD_{600} were chilled on ice, washed twice with growth medium containing 1 mg/ml BSA, and suspended in 100 μl of water, for lipid extraction, or in 500 μl of medium containing 1 mg/ml BSA, for use in a DHS release assay. In the DHS release assay, cells were incubated at 30°C for 10 min, and cells and medium were separated by centrifugation. Cells were then suspended in 100 μl of water. Lipids were extracted from both cells and medium by successive addition and mixing of 3.75 volumes of chloroform/methanol/HCl [100:200:1 (vol:vol)], 1.25 volumes of chloroform, and 1.25 volumes of 1% KCl. Phases were separated by centrifugation, and the organic phase was recovered, dried, and suspended in chloroform/methanol [2:1 (v/v)]. The labeled lipids were resolved by thin layer chromatography (TLC) on Silica Gel 60 high-performance TLC plates (Merck, Whitestation, NJ) with 1-butanol/acetic acid/water [3:1:1 (v/v)].

Table 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*Matα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.*, 1988
KHY13	SEY6210, Δ*dpl1*::*TRP1*	Kihara and Igarashi, 2002
KHY22	SEY6210, Δ*dpl1*::*TRP1* Δ*lcb4*::*LEU2*	This study
KHY288	SEY6210, Δ*dpl1*::*TRP1 lcb4*::mTn-*lacZ/LEU2*	This study
KHY294	SEY6210, Δ*dpl1*::*TRP1 yrm1*::mTn-*lacZ/LEU2*	This study
KHY299	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2*	This study
KHY312	SEY6210, Δ*dpl1*::*TRP1 dhh1*::mTn-*lacZ/LEU2*	This study
KHY323	SEY6210, Δ*dpl1*::*TRP1* Δ*pdr5*::*KanMX4*	This study
KHY360	SEY6210, Δ*dpl1*::*TRP1 LEU2*	This study
KHY364	SEY6210, Δ*dpl1*::*TRP1 LEU2* Δ*pdr5*::*KanMX4*	This study
KHY365	SEY6210, Δ*dpl1*::*TRP1 LEU2* Δ*dhh1*::*KanMX4*	This study
KHY396	SEY6210, Δ*dpl1*::*TRP1* Δ*pdr1*::*KanMX4*	This study
KHY400	SEY6210, Δ*dpl1*::*TRP1 LEU2 HIS3*	This study
KHY421	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2* Δ*yor1*::*KanMX4*	This study
KHY424	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2* Δ*rsb1*::*HIS3* Δ*yor1*::*KanMX4*	This study
KHY425	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2* Δ*yor1*::*KanMX4*	This study
KHY426	SEY6210, *rsb1*::*3xHA-RSB1*	This study
KHY427	SEY6210, Δ*dpl1*::*TRP1 LEU2* Δ*rsb1*::*HIS3*	This study
KHY444	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3*	This study
KHY452	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2 HIS3*	This study
KHY454	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2* Δ*rsb1*::*HIS3*	This study
KHY456	SEY6210, Δ*dpl1*::*TRP1 LEU2 HIS3* Δ*ros3*::*KanMX4*	This study
KHY457	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2 HIS3* Δ*ros3*::*KanMX4*	This study
KHY458	SEY6210, Δ*dpl1*::*TRP1 LEU2* Δ*rsb1*::*HIS3*	This study
KHY463	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*pdr1*::*KanMX4*	This study
KHY469	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr1*::*KanMX4*	This study
KHY515	SEY6210, *rsb1*::*3xHA-RSB1* Δ*yor1*::*HIS3*	This study
KHY531	SEY6210, *rsb1*::*3xHA-RSB1* Δ*ros3*::*HIS3*	This study
KHY532	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*ros3*::*HIS3*	This study
KHY533	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*yor1*::*HIS3*	This study
KHY579	SEY6210, *rsb1*::*3xHA-RSB1* Δ*dnf1*::*KanMX4*	This study
KHY583	SEY6210, *rsb1*::*3xHA-RSB1* Δ*dnf2*::*KanMX4*	This study
KHY590	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*yor1*::*HIS3* Δ*pdr1*::*KanMX4*	This study
KHY591	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*dnf2*::*KanMX4*	This study
KHY594	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*dnf1*::*KanMX4*	This study
KHY611	SEY6210, Δ*dpl1*::*TRP1 LEU2* Δ*dnf2*::*KanMX4* Δ*dnf1*::*HIS3*	This study
KHY612	SEY6210, *rsb1*::*3xHA-RSB1* Δ*dnf2*::*KanMX4* Δ*dnf1*::*HIS3*	This study
KHY614	SEY6210, Δ*dpl1*::*TRP1 pdr5*::mTn-*lacZ/LEU2* Δ*dnf2*::*KanMX4* Δ*dnf1*::*HIS3*	This study
KHY615	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*dnf2*::*KanMX4* Δ*dnf1*::*HIS3*	This study
KHY618	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr3*::*KanMX4*	This study
KHY619	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*pdr3*::*KanMX4*	This study
KHY620	SEY6210, *rsb1*::*3xHA-RSB1* Δ*pdr5*::*URA3* Δ*yor1*::*HIS3* Δ*pdr3*::*KanMX4*	This study

Immunoblotting

Immunoblotting was performed as described previously (Kihara and Igarashi, 2002) by using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) or Lumi-Light^{PLUS} Western blotting substrate (Roche Diagnostics, Mannheim, Germany). Anti-hemagglutinin (HA) (Y-11) antibody (0.2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Dpm1 antibody (2 μg/ml; Molecular Probes, Eugene, OR), anti-Pma1 (yN-20) antibody (0.4 μg/ml; Santa Cruz Biotechnology), horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG F(ab′)$_2$ fragment (1:7500 dilution; Amersham Biosciences), and HRP-conjugated donkey anti-goat IgG (0.08 μg/ml; Santa Cruz Biotechnology) were used.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared using the YeaStar RNA kit (Zymo Research, Orange, CA) according to the manufacturer's manual. The RNA was converted to cDNA by using the primer RSB1-R (5′-AATCGGGTATTTCATGCTTGCT-TGG-3′) and the ProSTAR first strand RT-PCR kit (Stratagene, La Jolla, CA). The *RSB1* cDNA was then amplified by PCR by using primers RSB1-F (5′-TTGTCATTTGGGGTATACTACTGAC-3′) and RSB1-R. RT-PCR for *ACT1* mRNA also was performed as a control by using the primer ACT1-R (5′-AACACTTGTGGTGAACGATAGATGG-3′) for preparing cDNA and primers ACT1-F (5′-GATTCTGGTATGTTCTAGCGCTTGC-3′) and ACT1-R for subsequent PCR.

Sucrose Gradient Fractionation

Cells grown in YPD medium were treated with 10 mM sodium azide. Approximately 4×10^8 cells were converted to spheroplast and lysed in 10% sucrose solution in buffer I (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1× protease inhibitor mixture [Complete; Roche Diagnostics, Indianapolis, IN], and 1 mM phenylmethylsulfonyl fluoride), by using an electric Potter homogenizer for 10 strokes. After removal of cell debris by centrifugation, total lysates (0.4 ml) were overlaid on a step sucrose gradient (0.4 ml of 55% sucrose, 1 ml of 45% sucrose, and 0.8 ml of 30% sucrose in buffer I) and centrifuged at 50,000 rpm (256,000 × g) in an S52ST rotor (Hitachi Koki, Tokyo, Japan) for 5 h. Fractions were collected from the top and separated by SDS-PAGE, followed by immunoblotting.

7-Nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-Lipid Trafficking Assay

Yeast cells were grown at 30°C in SC medium to 1 OD_{600} unit/ml, chilled on ice, and treated with 1/100 volume of 500 μM myristoyl-(NBD-hexanoyl)-PE (NBD-PE) (Avanti Polar Lipids, Alabaster, AL) or 500 μM myristoyl-(NBD-hexanoyl)-PC (NBD-PC) (Avanti Polar Lipids) while vigorously mixing. Cells were incubated at 4°C for 30 min and washed with cold SC medium. Cells assayed for flip were suspended in 100 μl water before lipid extraction. Cells being assayed for flop were further incubated in SC medium at 30°C for 0 or 30 min, washed with cold SC medium, and suspended in 100 μl of water.

Table 2. PHS sensitivity of the isolated mutants

		PHS[a] (μM)			
Strain	Genotype	7.5	10	12.5	15
KHY360	Δ*dpl1*	+++	++	±	−
KHY288	Δ*dpl1 lcb4*::mTn-*lacZ/LEU2*	+++	+++	+++	+++
KHY299	Δ*dpl1 pdr5*::mTn-*lacZ/LEU2*	+++	+++	+++	++
KHY312	Δ*dpl1 dhh1*::mTn-*lacZ/LEU2*	+++	+++	++	+
KHY294	Δ*dpl1 yrm1*::mTn-*lacZ/LEU2*	+++	+++	+	±
KHY22	Δ*dpl1* Δ*lcb4*	+++	+++	+++	+++
KHY364	Δ*dpl1* Δ*pdr5*	+++	+++	+++	++
KHY365	Δ*dpl1* Δ*dhh1*	+++	+++	++	+

[a] Cells were grown at 30°C on YPD plates containing PHS at the indicated concentrations and Nonidet P-40 [0.0015% (w/v), final concentration] as a dispersant. PHS sensitivity was evaluated as five grades (−, ±, +, ++, +++), according to colony size.

Lipids were extracted by the successive addition and mixing of 375 μl chloroform/methanol [1:2 (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 chloroform. The lipids were resolved by TLC on Silica Gel 60 high-performance TLC plates (Merck) with chloroform/methanol/28% NH_3/water [50:40:7:3 (v/v)]. NBD-lipids were quantified using a Bio Imaging analyzer FLA-2000 (Fuji Photo Film, Kanagawa, Japan).

RESULTS

Isolation of PHS-resistant, Transposon-inserted Mutants

Dpl1p is a LCBP lyase that degrades phytosphingosine 1-phosphate (PHS1P) to fatty aldehyde and phosphoethanolamine. In yeast cells, PHS is imported from the medium and converted into PHS1P by the LCB kinase Lcb4p. Overaccumulation of PHS1P is toxic to cells (Kim *et al*., 2000; Zhang *et al*., 2001), and Δ*dpl1* mutants are more sensitive to exogenous PHS than wild-type cells (Saba *et al*., 1997). Using this phenotype, we previously screened for a multicopy suppressor of the PHS-sensitive phenotype of the Δ*dpl1* mutants and obtained the *RSB1* gene (Kihara and Igarashi, 2002). We further demonstrated that this gene product is a putative LCB-transporter/translocase. In the present study, we searched for additional LCB/LCBP-related genes by using another approach, transposon mutagenesis.

The genomes of KHY13 (Δ*dpl1*) cells were randomly mutated by insertion of the mTn-*lacZ*/*LEU2* transposon. Pooled mutants were then plated on YPD medium, containing 15 μM PHS, and incubated at 30°C for 2 d. Several clones were obtained at a frequency of about 1 to 9000 mutants. Of these, 26 mutants were subjected to further analyses. To identify genes disrupted by the transposon, yeast genomic DNA adjacent to the transposon was cloned from each mutant. Sequence analyses revealed four classes of mutants, based on the genes interrupted by the transposon. Eighteen mutants contained the transposon insertion within the *LCB4* gene. Isolation of *lcb4* mutants had been expected, because Lcb4p is a major LCBP-synthesizing enzyme. Five mutants carried insertions in *PDR5*, the gene that encodes the ABC transporter Pdr5p, which reportedly confers resistance to a wide range of compounds (Rogers *et al*., 2001). Two others were mutants of the *DHH1* gene, which encodes a putative RNA helicase that acts as a component of a CCR4-NOT transcriptional regulatory factor complex (Hata *et al*., 1998; Maillet and Collart, 2002). One mutation was inserted by the transposon in the *YRM1* gene, normally encoding a transcription factor (Lucau-Danila *et al*., 2003).

Among the isolated mutants, the *lcb4* mutants exhibited the strongest phenotype with regard to PHS resistance. Control KHY360 (Δ*dpl1*) cells (KHY13 cells carrying a wild-type *LEU2* gene to control for the *LEU2* transposon) were not able to grow in the presence of 15 μM PHS, yet an *lcb4* mutant, KHY288, was not similarly inhibited (Table 2). Additionally, the *pdr5* mutants were highly resistant to PHS. Although their growth rate in 15 μM PHS was slightly reduced, that at 12.5 μM was not affected (Table 2). In contrast, the PHS resistance of the *dhh1* mutants was rather weak, because their growth in 15 μM PHS was obviously retarded (Table 2). The *yrm1* mutants, KHY294, were the most sensitive to PHS of the obtained mutants, with only a slight difference in the sensitivity between them and the KHY360 cells (Table 2).

We next constructed deletion mutants for *LCB4*, *PDR5*, *DHH1*, or *YRM1* in the Δ*dpl1* background and investigated their PHS sensitivity. KHY22 (Δ*dpl1* Δ*lcb4*), KHY364 (Δ*dpl1* Δ*pdr5*), and KHY365 (Δ*dpl1* Δ*dhh1*) cells exhibited PHS resistance identical to that of their respective transposon mutants (Table 2), indicating that their gene disruptions were indeed responsible for the PHS resistance. However, the Δ*dpl1* Δ*yrm1* mutants did not show any PHS resistance (our unpublished data). For further analyses we used the *pdr5* mutants, because these exhibited the most striking PHS sensitivity, excluding the already characterized lcb4p.

Efflux of LCB Is Enhanced in pdr5 Mutants

We first investigated whether the *pdr5* mutation affects the intracellular accumulation of LCBs. KHY13 (*PDR5*$^+$), KHY299 [*pdr5* (*pdr5*::mTn-*lacZ/LEU2*)], and KHY323 (Δ*pdr5*) cells were labeled with [^{3}H]DHS for 5, 20, and 60 min at 30°C. Then, intracellularly accumulated DHS was extracted, separated by TLC, and quantified. As shown in Figure 1, wild-type cells accumulated DHS in a time-dependent manner, so that ~40% of the added DHS had accumulated intracellularly by 60 min. On the other hand, accumulation of [^{3}H]DHS in the Δ*pdr5* mutants was reduced, to ~70% of that in wild-type cells (Figure 1). Likewise, the transposon-inserted mutant KHY299 exhibited a decrease in the DHS accumulation similar to that of the *pdr5* deletion mutants (Figure 1).

Reduced accumulation of DHS could be caused by a decrease in its uptake or an increase in its efflux. To examine the second possibility, we next performed a DHS release assay. Wild-type and Δ*pdr5* cells were labeled with [^{3}H]DHS for 1 h at 30°C to allow cells to accumulate DHS. Cells were then washed and incubated for 10 min at 30°C. Lipids were extracted from both media and cells and separated by TLC. Wild-type cells released 7% of the accumulated DHS,

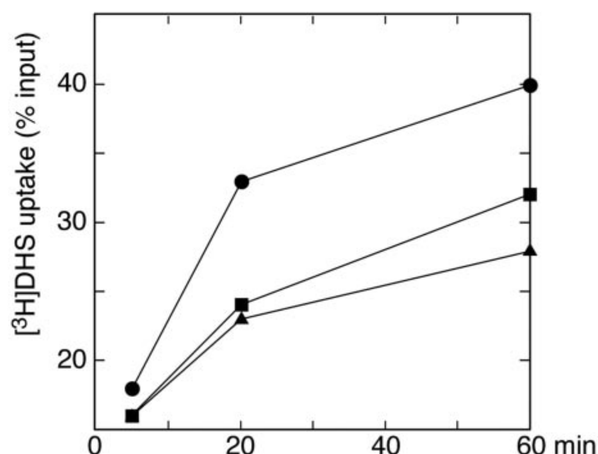

Figure 1. DHS accumulation decreases in *pdr5* mutants. KHY13 (*PDR5*$^+$; circles), KHY299 (*pdr5*::mTn-*lacZ/LEU2*; triangles), and KHY323 (Δ*pdr5*; squares) cells grown in YPD medium were labeled with [^{3}H]DHS complexed with BSA, for 5, 20, and 60 min. Cellular lipids were extracted and separated by TLC. Radioactivities associated with DHS were each quantified and are expressed as a percentage of input radioactivity.

whereas the Δ*pdr5* cells exported 22% (Figure 2), indicating increased DHS release.

RSB1 Is Involved in Enhanced LCB Export Caused by the pdr5 Mutation

The possibility that Pdr5p normally acts as an efflux pump for DHS was deemed unlikely, because the DHS release would have been reduced, not increased, by the *pdr5* mutation. Instead, the effect seemed to be indirect. Rsb1p would be a likely candidate for a protein influenced by a *pdr5* mutation, because we recently identified it as a putative transporter/translocase for LCBs including PHS, DHS, and sphingosine (Kihara and Igarashi, 2002). To investigate this hypothesis, we created Δ*dpl1* Δ*rsb1* mutants and Δ*dpl1 pdr5* Δ*rsb1* mutants. KHY427 cells (Δ*dpl1* Δ*rsb1*) were slightly more sensitive to PHS than KHY400 cells (Δ*dpl1*) (Table 3), due to a reduction in the efflux of PHS (Kihara and Igarashi, 2002; Figure 2). Although KHY452 cells (Δ*dpl1 pdr5*) were resistant to PHS, as described above, introducing the Δ*rsb1* mutation caused the resulting cells (KHY454) to become as sensitive to PHS as the KHY427 cells (Table 3). This indicated that the PHS-resistant phenotype of the *pdr5* mutants was completely dependent on *RSB1*. Furthermore, the [^{3}H]DHS release assay demonstrated that the efflux, unequivocally exhibited by wild-type cells (7.5%), was greatly reduced in the Δ*rsb1* mutant (0.9%) (Figure 2). Thus, Rsb1p is responsible for most of the DHS export activity in wild-type cells. Consistent with their PHS sensitivity, the *pdr5* Δ*rsb1* cells export only slight amounts of DHS (1.3%) that are almost identical to those released by the Δ*rsb1* mutant (Figure 2).

We next searched for other ABC transporter genes involved in the PHS resistance, together with the *PDR5* gene. For this purpose, we introduced several deletion mutations into KHY452 (Δ*dpl1 pdr5*) cells and investigated their PHS sensitivity. These mutations included Δ*pdr10*, Δ*pdr12*, Δ*pdr15*, Δ*pdr18*, Δ*snq2*, and Δ*yor1*. Of these, only KHY425 (Δ*dpl1 pdr5* Δ*yor1*) cells exhibited enhanced PHS resistance, compared with the KHY452 cells (Table 3; our unpublished

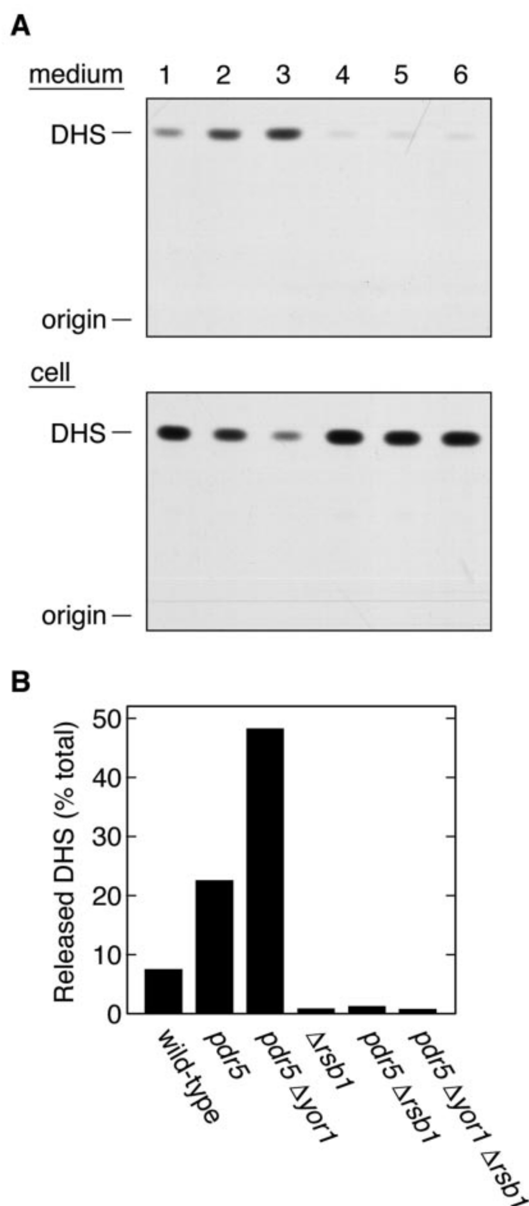

Figure 2. DHS release is increased in *pdr5* mutants in an *RSB1*-dependent manner. (A) KHY400 (wild-type; lane 1), KHY452 (*pdr5*; lane 2), KHY425 (*pdr5* Δ*yor1*; lane 3), KHY427 (Δ*rsb1*; lane 4), KHY454 (*pdr5* Δ*rsb1*; lane 5), and KHY424 (*pdr5* Δ*yor1* Δ*rsb1*; lane 6) cells were grown in YPD medium and incubated with [^{3}H]DHS complexed with BSA, for 1 h at 30°C. The cells were then washed and suspended in YPD medium containing BSA. After incubating for 10 min at 30°C, the media were separated from the cells by centrifugation. Lipids were extracted and separated by TLC for both media (4/5 total lipids; top) and cells (one-third total lipids; bottom). (B) Radioactivities associated with the DHS recovered in the media are each expressed as a percentage of the total radioactivity from both cells and media.

data). However, Δ*dpl1* Δ*yor1* mutants were not PHS resistant (our unpublished data), indicating that the Δ*yor1* mutation was effective only when combined with the *pdr5* mutation. Additionally, the DHS release assay demonstrated that the *pdr5* Δ*yor1* mutants exported approximately twofold more DHS than the *pdr5* mutants did (Figure 2), confirming that

Table 3. The PHS-resistant phenotype of the *pdr5* mutant is dependent on *RSB1*

		PHS[a] (μM)				
Strain	Genotype	7.5	10	12.5	15	17.5
KHY400	Δ*dpl1*	+++	++	±	−	−
KHY452	Δ*dpl1 pdr5*	+++	+++	+++	++	±
KHY425	Δ*dpl1 pdr5* Δ*yor1*	+++	+++	+++	+++	++
KHY427	Δ*dpl1* Δ*rsb1*	+++	+	−	−	−
KHY454	Δ*dpl1 pdr5* Δ*rsb1*	+++	+	−	−	−
KHY424	Δ*dpl1 pdr5* Δ*yor1* Δ*rsb1*	+++	+	−	−	−

[a] Cells were grown at 30°C on YPD plates containing PHS at the indicated concentrations and Nonidet P-40 [0.0015% (w/v), final concentration]. PHS sensitivity was evaluated as five grades (−, ±, +, ++, +++), according to colony size.

the enhanced PHS resistance caused by the Δ*yor1* mutation was due to an increased efflux of PHS. The PHS-resistant phenotype of the Δ*dpl1 pdr5* Δ*yor1* cells was again *RSB1* dependent, because KHY424 (Δ*dpl1 pdr5* Δ*yor1* Δ*rsb1*) cells were sensitive to PHS (Table 3), and the export rate of DHS by the *pdr5* Δ*yor1* Δ*rsb1* mutants was very low (Figure 2).

Up-Regulation of Rsb1p in Δpdr5 and Δpdr5 Δyor1 Mutants

We next considered whether the enzyme activity or the amount of Rsb1p was increased in the *pdr5* and *pdr5* Δ*yor1* mutants. To examine the enzyme activity of Rsb1p, we introduced the pAK170 plasmid, encoding N-terminally, triple HA (3xHA)-tagged *RSB1* (*3xHA-RSB1*), into both the KHY458 (Δ*rsb1*) and KHY424 (Δ*rsb1 pdr5* Δ*yor1*) cells. Because this *3xHA-RSB1* fusion gene was designed to be expressed constitutively under the control of the *TDH3* promoter, the amounts of Rsb1p were expected to be constant among the cells. Indeed, immunoblotting by using an anti-HA antibody demonstrated that KHY458 cells bearing pAK170 expressed 3xHA-Rsb1p at levels nearly equal to those of KHY424 cells bearing pAK170 (Figure 3A). Introduction of the pAK170 plasmid into the KHY458 cells resulted in an ~12-fold increase in DHS release; a similar increase also was observed in the KHY424 cells bearing pAK170 (Figure 3B). These results suggested that the activity per Rsb1p molecule was not affected in the *pdr5* Δ*yor1* cells. We also looked for differences in the intracellular localization of Rsb1p between the *pdr5* and *pdr5* Δ*yor1* mutants, but we found that the sucrose gradient fractionation profiles of 3xHA-Rsb1p expressed in wild-type and in the *pdr5* Δ*yor1* cells were similar (our unpublished data).

Therefore, we next examined the possibility that the amount of Rsb1p was increased in the *pdr5* and *pdr5* Δ*yor1* cells. To detect endogenous Rsb1p, the *RSB1* gene was replaced with *3xHA-RSB1*, with the *RSB1* promoter remaining intact, creating KHY426 cells. KHY444 (Δ*pdr5*), KHY515 (Δ*yor1*), and KHY533 (Δ*pdr5* Δ*yor1*) cells were then produced by introducing the Δ*pdr5* and/or Δ*yor1* mutations into the KHY426 cells. Immunoblotting by using an anti-HA antibody demonstrated that 3xHA-Rsb1p was increased in the Δ*pdr5* cells compared with the control KHY426 cells (Figure 4A). Although the Δ*yor1* single mutation did not affect the amount of 3xHA-Rsb1p, the Δ*pdr5* Δ*yor1* double mutation further enhanced the expression of 3xHA-Rsb1p compared with the Δ*pdr5* single mutation (Figure 4A). These protein amounts correlated well with the PHS sensitivity (Table 3) and the DHS export activity of the respective mutations (Figure 2).

To investigate whether the increases in Rsb1p amounts in the *pdr5* and *pdr5* Δ*yor1* cells resulted from changes at the transcriptional level, we performed an RT-PCR analysis. Total RNAs were isolated from KHY400 (wild-type), KHY452 (*pdr5*), and KHY425 (*pdr5* Δ*yor1*) cells and subjected to RT-PCR by using primers specific for *RSB1*, and for *ACT1*, encoding actin, as a control. As shown in Figure 4B, the amount of *RSB1* amplified from RNA prepared from the *pdr5* mutants was increased compared with that from wild-

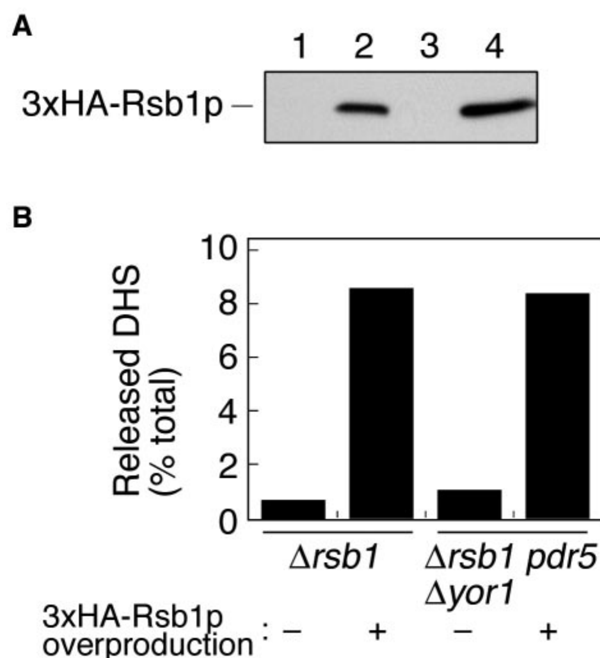

Figure 3. The enzyme activity of Rsb1p is unchanged in *pdr5* Δ*yor1* cells. (A) KHY458 cells (Δ*rsb1*) bearing pAK80 (vector) (lane 1) or pAK170 (*3xHA-RSB1*) (lane 2) and KHY424 cells (Δ*rsb1 pdr5* Δ*yor1*) bearing pAK80 (lane 3) or pAK170 (lane 4) were grown at 30°C in SC medium lacking uracil. (A) Total proteins from cell lysates were separated by SDS-PAGE and detected by immunoblotting with anti-HA antibodies. (B) Cells were labeled with [^{3}H]DHS complexed with BSA for 1 h at 30°C, and then washed and suspended in medium containing BSA. After incubating for 10 min at 30°C, the media were separated from the cells by centrifugation. Lipids were extracted from both media and cells and separated by TLC. Radioactivities associated with the DHS recovered in the media are each expressed as a percentage of the total radioactivity from both cells and media.

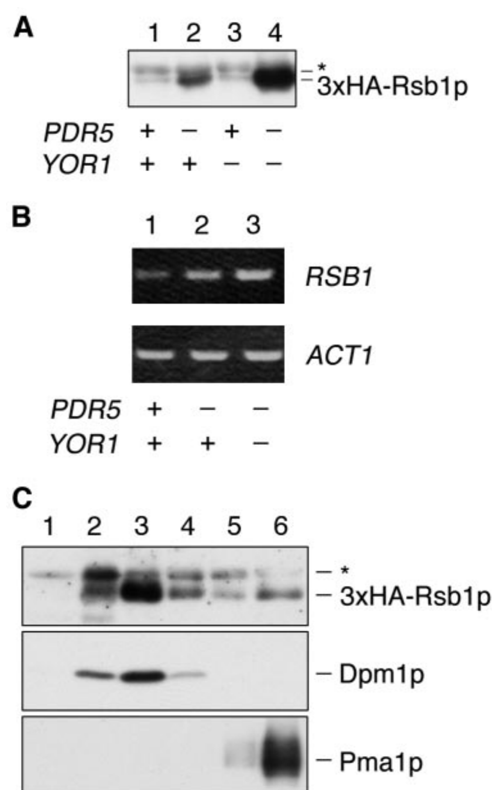

Figure 4. Expression of *RSB1* is increased in Δ*pdr5* and Δ*pdr5* Δ*yor1* cells. (A) KHY426 (wild-type; lane 1), KHY444 (Δ*pdr5*; lane 2), KHY515 (Δ*yor1*; lane 3), and KHY533 (Δ*pdr5* Δ*yor1*; lane 4) cells were grown in YPD medium at 30°C. Total cell lysates were prepared, and fixed amounts of proteins (25 μg) were subjected to immunoblotting by using anti-HA antibodies. (B) Total RNA was prepared from KHY400 (wild-type; lane 1), KHY452 (*pdr5*; lane 2), and KHY425 (*pdr5* Δ*yor1*; lane 3) cells and subjected to RT-PCR by using *RSB1*- and *ACT1*-specific primers. (C) Total cell lysates prepared from KHY533 cells were fractionated by sucrose gradient centrifugation. Samples were collected from the top (fraction 1) to the bottom (fraction 6) of the gradient. Proteins were separated by SDS-PAGE and subjected to immunoblotting by using anti-HA, anti-Dpm1p, and anti-Pma1p antibodies. The asterisks indicate nonspecific background.

type cells. Moreover, the *pdr5* Δ*yor1* double mutations exhibited an even higher amount (Figure 4B). These results clearly showed that Rsb1p is up-regulated in the *pdr5* and *pdr5* Δ*yor1* mutants at a transcriptional level.

In a previous study, we investigated the intracellular localization of 3xHA-Rsb1p by using immunofluorescence microscopy and found that 3xHA-Rsb1p is localized at both the ER and the plasma membrane (Kihara and Igarashi, 2002). However, in that study, 3xHA-Rsb1p was overproduced under the control of the *TDH3* promoter, and its distribution between the ER and the plasma membrane was not quantitatively assessed. Therefore, we investigated the localization in KHY533 cells expressing 3xHA-Rsb1p under its own promoter, by using sucrose gradient fractionation. As shown in Figure 4C, 3xHA-Rsb1p exhibited two peaks: a major peak in fraction 3 and a minor peak in fraction 6. The ER membrane protein Dpm1p and the plasma membrane protein Pma1p were highest in fractions 3 and 6, respectively. These results indicated that 3xHA-Rsb1p resides both at the ER and the plasma membrane, with the majority at the ER.

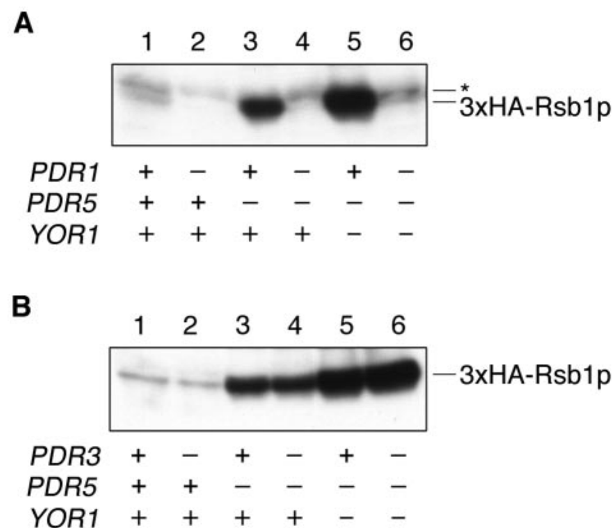

Figure 5. Transcription of the *RSB1* gene is under the control of Pdr1p but not Pdr3p. Cells were grown in YPD medium at 30°C, and proteins were prepared from cell lysates. Total proteins (25 μg) were separated by SDS-PAGE, followed by detection with anti-HA antibodies. (A) KHY426 (wild-type; lane 1), KHY469 (Δ*pdr1*; lane 2), KHY444 (Δ*pdr5*; lane 3), KHY463 (Δ*pdr5* Δ*pdr1*; lane 4), KHY533 (Δ*pdr5* Δ*yor1*; lane 5), and KHY590 (Δ*pdr5* Δ*yor1* Δ*pdr1*; lane 6) cells were used. (B) KHY426 (lane 1), KHY618 (Δ*pdr3*; lane 2), KHY444 (lane 3), KHY619 (Δ*pdr5* Δ*pdr3*; lane 4), KHY533 (lane 5), and KHY620 (Δ*pdr5* Δ*yor1* Δ*pdr3*; lane 6) cells were used. The asterisk indicates nonspecific background.

Expression of RSB1 Is Under the Control of Pdr1p

Pdr1p and Pdr3p are zinc finger-type transcription factors. Their gain-of-function mutations, such as the *PDR1-3* and *PDR3-7* mutations, cause cells to acquire a pleiotropic drug-resistant phenotype through the activation of several ABC transporter family members (Balzi *et al*., 1994; Carvajal *et al*., 1997; Nourani *et al*., 1997; DeRisi *et al*., 2000). Previous comprehensive microarray analysis investigating genes up-regulated by the *PDR1-3* and/or *PDR3-7* mutation revealed that the expression of *RSB1* was enhanced, markedly by the *PDR1-3* mutation and slightly by the *PDR3-7* mutation (DeRisi *et al*., 2000). Therefore, we investigated roles for Pdr1p and Pdr3p in the transcriptional regulation of the *RSB1* gene, by using their disruptants. When the Δ*pdr1* mutation was introduced into KHY426 (*3xHA-RSB1*) cells, no expression of 3xHA-Rsb1p was detected in the lysate of the resulting strains (KHY469) by immunoblotting (Figure 5A). The increased expression of Rsb1p observed in the Δ*pdr5* and Δ*pdr5* Δ*yor1* cells also was diminished by the introduction of the Δ*pdr1* mutation (Figure 5A), indicating that the expression of Rsb1p was under the control of Pdr1p. In contrast, the Δ*pdr3* mutation had no effect on the Rsb1p expression in either background tested (*PDR5*$^+$ *YOR1*$^+$, *pdr5 YOR1*$^+$, or *pdr5* Δ*yor1*) (Figure 5B).

There are two TCCGCGGA nucleotide sequences in the *RSB1* gene that match the pleiotropic drug response elements (PDREs; TCCG/AC/TGG/CA/G), situated 816 base pairs and 758 base pairs upstream of the translational initiation codon. We designated these sequences PDRE-1 (−816 to −809 from the start of the *RSB1* gene) and PDRE-2 (−758 to −751). Because the PDREs are known to be recognized by Pdr1p (Katzmann *et al*., 1996), we investigated whether PDRE-1 and PDRE-2 were required for the influence of Pdr1p on the expression of *RSB1*. For this purpose, we

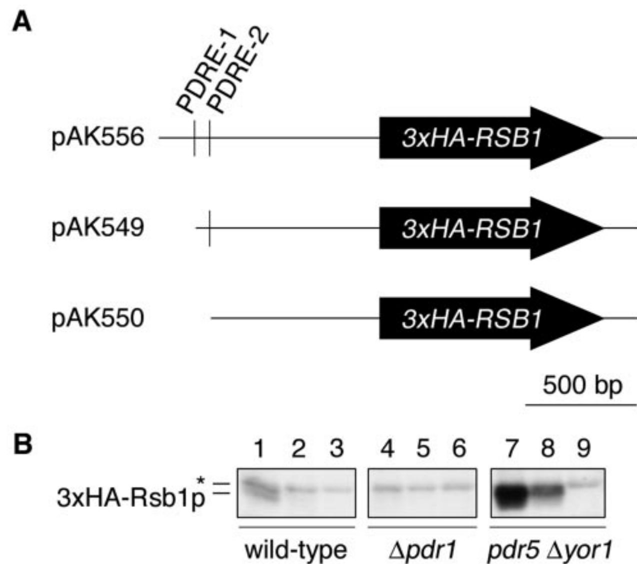

Figure 6. Both PDREs are required for expression of the *RSB1* gene. (A) Schematic representation of the positions of PDRE-1 and PDRE-2 and the relative lengths of the constructed plasmids. (B) The plasmids pAK556 (lanes 1, 4, and 7), pAK549 (lanes 2, 5, and 8), and pAK550 (lanes 3, 6, and 9) were introduced into KHY13 (wild-type; lanes 1–3), KHY396 (Δ*pdr1*; lanes 4–6), and KHY421 (*pdr5* Δ*yor1*; lanes 7–9) cells. Total lysates were prepared from each cell type, and fixed amounts of protein (25 μg) were separated by SDS-PAGE. 3xHA-Rsb1p was visualized by immunoblotting by using anti-HA antibodies.

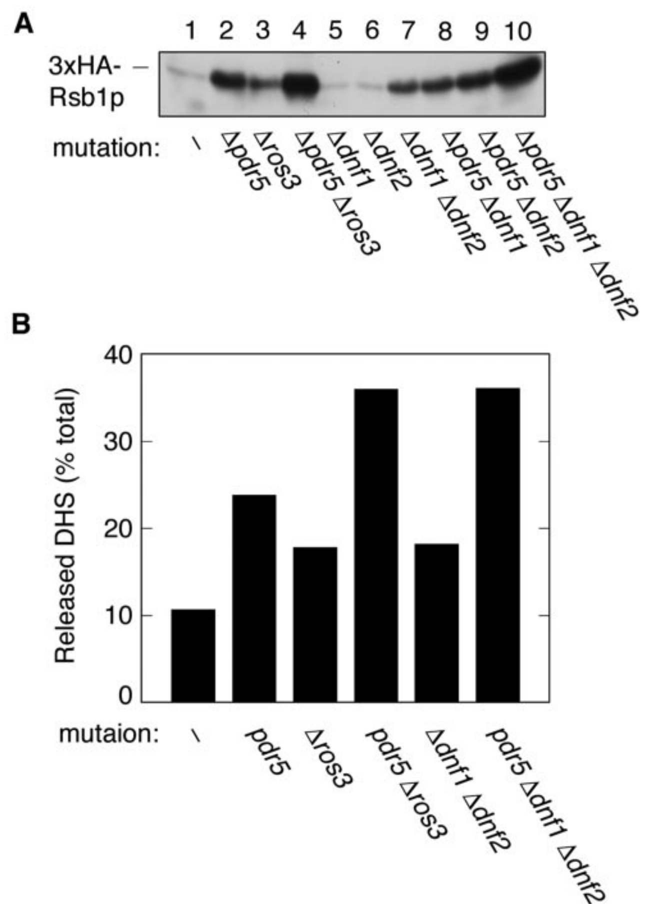

Figure 7. The amounts of Rsb1p are increased in Δ*ros3* and Δ*dnf1* Δ*dnf2* cells. (A) KHY426 (wild-type; lane 1), KHY444 (Δ*pdr5*; lane 2), KHY531 (Δ*ros3*; lane 3), KHY532 (Δ*pdr5* Δ*ros3*; lane 4), KHY579 (Δ*dnf1*; lane 5), KHY583 (Δ*dnf2*; lane 6), KHY612 (Δ*dnf1* Δ*dnf2*; lane 7), KHY594 (Δ*pdr5* Δ*dnf1*; lane 8), KHY591 (Δ*pdr5* Δ*dnf2*; lane 9), and KHY615 (Δ*pdr5* Δ*dnf1* Δ*dnf2*; lane 10) cells were grown in YPD medium. Total proteins (25 μg) prepared from each were separated by SDS-PAGE, followed by immunoblotting by using anti-HA antibodies. (B) KHY400 (wild-type), KHY452 (*pdr5*), KHY456 (Δ*ros3*), KHY457 (*pdr5* Δ*ros3*), KHY611 (Δ*dnf1* Δ*dnf2*), and KHY614 (*pdr5* Δ*dnf1* Δ*dnf2*) cells grown in YPD medium were labeled with [^{3}H]DHS complexed with BSA for 1 h at 30°C. After being washed, the cells were suspended in YPD medium containing BSA and incubated for 10 min at 30°C. The media were separated from the cells by centrifugation. Lipids were extracted from both media and cells and separated by TLC. Radioactivities associated with the DHS recovered in the media are each expressed as a percentage of the total radioactivity from both cells and media.

constructed three plasmids (pAK556, pAK549, and pAK550) of different lengths, carrying nucleotides found 5′-upstream of the *RSB1* gene fused to the *3xHA-RSB1* construct, as illustrated in Figure 6A. 3xHA-Rsb1p was detected when pAK556, which contained both PDRE-1 and PDRE-2, was introduced into wild-type cells (Figure 6B, lane 1). However, its expression was not detected in KHY396 cells (Δ*pdr1*) bearing pAK556 (Figure 6B, lane 4). In contrast, a marked increase in the amount of 3xHA-Rsb1p was observed in KHY421 cells (*pdr5* Δ*yor1*) harboring pAK556 (Figure 6B, lane 7). Expression of 3xHA-Rsb1p from pAK549, which carries only PDRE-2, was decreased both in wild-type and *pdr5* Δ*yor1* cells, compared with that from pAK556 (Figure 6B). Moreover, pAK550, which lacks both PDRE-1 and PDRE-2, could not drive the 3xHA-Rsb1p expression in either cell type (Figure 6B). Thus, both PDRE-1 and PDRE-2 are required for full expression of *RSB1*.

Expression of Rsb1p Is Increased in Δros3 and Δdnf1 Δdnf2 Cells

Pdr5p and Yor1p are not only involved in drug efflux but also in translocation of PE (Decottignies *et al*., 1998). High levels of Rsb1p were observed in Δ*pdr5* and Δ*pdr5* Δ*yor1* cells. However, because this occurred in the absence of drug treatment, we assumed that altered glycerophospholipid asymmetry was the cause of the high expression. Glycerophospholipid asymmetry is maintained by both P-type ATPase-dependent inward movement (flip) and by ABC transporter-dependent outward movement (flop). Therefore, we next examined whether the expression of Rsb1p would be increased by a reduced inward movement of glycerophospholipids. For this purpose, we disrupted two P-type ATPase-encoding genes, *DNF1* and *DNF2*, in the *3xHA-RSB1* cells. Although neither a Δ*dnf1* nor Δ*dnf2* single mutation caused an increase in the amount of 3xHA-Rsb1p in either the *PDR5*$^+$ or Δ*pdr5* background, Δ*dnf1* Δ*dnf2* double mutants exhibited a marked accumulation of 3xHA-Rsb1p (Figure 7A). A greater increase was observed in the Δ*pdr5* Δ*dnf1* Δ*dnf2* mutants (Figure 7A). The recently identified *ROS3* gene is involved in the flip of glycerophospholipid (Kato *et al*., 2002; Hanson *et al*., 2003). As shown in Figure 7A, the amount of 3xHA-Rsb1p also was increased in Δ*ros3* mutants and further elevated in Δ*ros3* Δ*pdr5* mutants.

The [^{3}H]DHS release assay demonstrated that the amount of exported DHS was increased in both the Δ*ros3* and Δ*dnf1* Δ*dnf2* mutants, compared with the wild-type cells (Figure 7B). Moreover, a further increase in the DHS release was

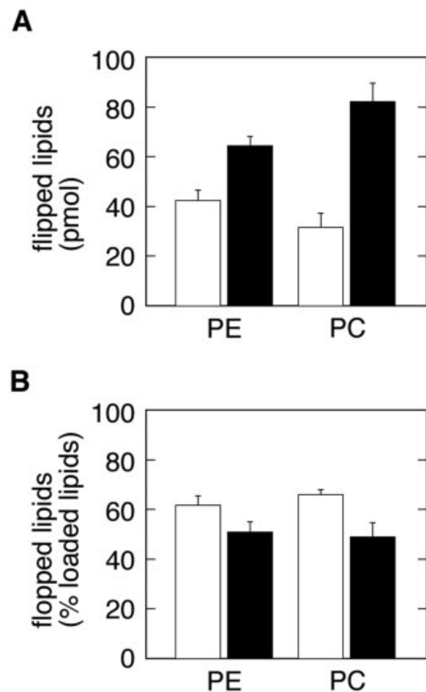

Figure 8. Overproduction of Rsb1p affects the flip-flop of NBD-labeled glycerophospholipids. KHY13 cells bearing pAK80 (vector; open columns) or pAK90 (*RSB1*; closed columns) plasmids were grown in SC medium lacking uracil at 30°C. (A) The cells were chilled on ice and treated with 5 μM NBD-PE or 5 μM NBD-PC for 30 min at 4°C. After the cells were washed, lipids were extracted and separated by TLC. Labeled lipids were quantified using a Bio Imaging analyzer FLA-2000. (B) Cells labeled as in A were washed and further incubated in SC medium at 30°C for 0 or 30 min. Cells were then washed, and lipids were extracted, separated by TLC, and quantified as in A. Values presented are the differences between the levels of the labeled lipids between before (0 min) and after incubation (30 min) as a percentage of the lipids loaded (0 min). All values represent a mean ± SD from three independent experiments.

observed in the *pdr5* Δ*ros3* and *pdr5* Δ*dnf1* Δ*dnf2* cells (Figure 7B). Thus, the intracellular levels of Rsb1p correlated well with the amounts of released DHS.

Overproduction of Rsb1p Affects the Glycerophospholipid Translocation

The results mentioned above suggested that altered glycerophospholipid asymmetry modulates the distribution of LCBs between the two leaflets of the lipid bilayer by increasing the expression of Rsb1p. We investigated the possibility that altered LCB asymmetry resulting from changes in the Rsb1p levels affects the trans-bilayer movement of glycerophospholipids, by using the recently established flip-flop assay (Kean *et al.*, 1997; Hanson and Nichols, 2001). Wild-type cells bearing the vector plasmid (pAK80) or those bearing the pAK90 plasmid that overproduces Rsb1p were incubated with NBD-PE or NBD-PC at 4°C for 30 min, allowing only the flip but not the flop reaction. For the flop assay, cells loaded with either lipid were further incubated at 30°C for 30 min. As shown in Figure 8A, cells overproducing Rsb1p flipped more NBD-PE (1.5-fold) and NBD-PC (2.6-fold) than control cells. On the other hand, flopping rates for both lipids were slightly reduced in cells overexpressing Rsb1p compared with cells bearing the vector plasmid (Figure 8B). These differences in the NBD-lipid flip/flop rates were not due to different sensitivities in these cells. Cells treated either with DMSO, 5 μM NBD-PE, or 5 μM NBD-PC at 4°C for 30 min were washed with medium, diluted, and incubated on SD-URA plates at 30°C. There were no differences in colony number or colony size. Indeed, the NBD-lipids exhibited no toxicity, at least at the concentrations used (our unpublished data). Thus, altered LCB asymmetry caused by overproduced Rsb1p affected the glycerophospholipid translocation, supporting the contention that alternations in the lipid asymmetry of glycerophospholipids and LCBs modulate the lipid distribution in a mutual manner.

DISCUSSION

Glycerophospholipids in the plasma membrane are arranged asymmetrically between the two leaflets of the lipid bilayer. In erythrocytes, the best characterized system, PS, PE, and PI, are abundant in the inner leaflet, whereas PC is mainly located in the outer leaflet (Schroit and Zwaal, 1991). Recent studies have revealed that certain P-type ATPases and ABC transporters are involved in their inward and outward movements, respectively (Bevers *et al.*, 1999; Williamson and Schlegel, 2002). On the other hand, reports concerning factors regulating the topology of sphingolipids are very limited. We previously identified the *RSB1* gene, which suppresses the LCB-sensitive phenotype of the Δ*dpl1* mutants when introduced as a multicopy plasmid (Kihara and Igarashi, 2002). Subsequent studies suggested that Rsb1p is an ATP-dependent LCB transporter or translocase (Kihara and Igarashi, 2002). Because LCBs are extremely hydrophobic and do not dissolve in aqueous solutions, LCBs added to culture medium may be incorporated spontaneously into the outer leaflet of the plasma membrane then be translocated to the inner leaflet by an unknown translocase. It is not clear whether Rsb1p is a transporter that pumps LCBs from the inner leaflet of the plasma membrane directly to the external medium, or a translocase (floppase) that flops LCBs from the inner leaflet to the outer leaflet. However, even if Rsb1p functions as a transporter, the released LCBs are likely then reincorporated into the plasma membrane, as shown previously (Kihara and Igarashi, 2002); thus, their population in the outer leaflet is increased as well. So, whether Rsb1p is a transporter or a translocase, increases in Rsb1p would likely lead to the same result: changes in the asymmetry of LCB in the plasma membrane. In the medium used for the [^{3}H]DHS release assay we included BSA, which has a high affinity for lipids. In the absence of BSA, the released DHS measured in the media was greatly reduced (our unpublished data). If Rsb1p is a translocase, LCBs flopped by Rsb1p toward the outer leaflet of the plasma membrane might be extracted by the exogenous BSA.

We favor the scenario that Rsb1p is a translocase rather than a transporter, because the majority of Rsb1p is localized at the ER (Figure 4C). This localization is reasonable, because its substrate LCBs are synthesized at the ER. In physiological conditions, Rsb1p may function to flop LCBs at the ER. Because lipids synthesized at the ER are transported to the plasma membrane via the Golgi apparatus by vesicular transport, without the loss of their membrane topology, the localization of the LCBs in the outer leaflet of the plasma membrane is likely established already at the ER. Therefore, in the [^{3}H]DHS release assay, it is possible that the imported DHS is first transported to the ER, where it is translocated to the extracytoplasmic leaflet by ER-resident Rsb1p and then delivered to the plasma membrane. The other possibility is that the plasma membrane-localized Rsb1p flops the imported DHS to the outer leaflet of the plasma membrane.

Indeed, LCBs are rapidly transported from the plasma membrane to the ER by either of two pathways. In the first pathway, LCBs are converted to LCBPs by the LCB kinase Lcb4p and then reconverted to LCBs at the ER by the LCBP phosphatase Lcb3p (Qie *et al*., 1997; Mao *et al*., 1999; Zanolari *et al*., 2000; Funato *et al*., 2003). In the second pathway, LCBs are directly transferred to the ER without conversion to LCBPs (Funato *et al*., 2003). Although previous studies have shown that the first pathway is the major pathway (Qie *et al*., 1997; Mao *et al*., 1999; Zanolari *et al*., 2000), the contribution of the second pathway is important in the yeast backgrounds used here, because exogenously added [^{3}H]DHS is effectively metabolized at the ER in the Δ*lcb4* cells (our unpublished data).

Although overexpression of Rsb1p affected the trans-bilayer movement of glycerophospholipids (Figure 7), deletion of the *RSB1* gene had no effect (our unpublished data). Because the expression level of Rsb1p is very low in wild-type cells (Figure 4A), its activity also may remain low, at least under the growth conditions used here, which may explain the lack of any effect from the deletion. We speculate that in certain conditions affecting the glycerophospholipid asymmetry, Rsb1p is induced and begins to function.

In this study, we demonstrated that the expression of Rsb1p is increased in cells with altered glycerophospholipid asymmetry due to the disruption of either the inward or outward movement of glycerophospholipids (Figures 4 and 7). Moreover, overexpression of Rsb1p affected both the inward and outward movements of glycerophospholipids (Figure 8). Thus, there may be a mechanism that maintains proper lipid asymmetry by regulating the trans-bilayer movement of both glycerophospholipids and sphingolipids. A mutant (*end8*) of *LCB1*, which normally encodes a subunit of a serine palmitoyltransferase involved in the first step of sphingolipid synthesis, was isolated and found to exhibit endocytosis defects (Zanolari *et al*., 2000). LCBs, it was found, are required for endocytosis (Zanolari *et al*., 2000). Certain P-type ATPase mutants also exhibit endocytosis defects (Pomorski *et al*., 2003). Thus, glycerophospholipids and LCBs seem to function together in some cellular events occurring at the plasma membrane. We speculate that cells with improper glycerophospholipid asymmetry alter the membrane distribution of LCBs between the inner and outer leaflets by increasing the expression of Rsb1p, to compensate for certain decreased plasma membrane functions such as endocytosis and cell polarity.

This study revealed the existence of cross talk between glycerophospholipids and sphingolipids in establishing membrane asymmetry. Pdr1p seems to play an important role in this cross talk, because not only Rsb1p but also Pdr5p and Yor1p are under its control (Meyers *et al*., 1992; Katzmann *et al*., 1995). However, how signals of altered membrane asymmetry affect Pdr1p is largely unknown. As an intriguing model, we propose that an unidentified sensor protein recognizing the glycerophospholipid asymmetry transduces the signal to Pdr1p. Although Rsb1p is regulated at the transcriptional level, RT-PCR experiments demonstrated that the altered trans-bilayer movement of glycerophospholipids caused by the overproduction of Rsb1p was not due to changes in the mRNA levels of *DNF1*, *DNF2*, *PDR5*, or *YOR1* (our unpublished data). Thus, the protein stability, activity, or intracellular localization of these enzymes may be affected by the change in the LCB asymmetry. However, further studies are required to reveal the precise molecular mechanism.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Snyder for providing mTn-*lacZ/LEU2*-mutagenized yeast genomic library. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (B) (12140201) and a Grant-in-Aid for Young Scientists (B) (15770078) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

Balzi, E., Wang, M., Leterme, S., Van Dyck, L., and Goffeau, A. (1994). PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. J. Biol. Chem. *269*, 2206–2214.

Bevers, E.M., Comfurius, P., Dekkers, D.W., and Zwaal, R.F. (1999). Lipid translocation across the plasma membrane of mammalian cells. Biochim. Biophys. Acta *1439*, 317–330.

Burns, N., Grimwade, B., Ross-Macdonald, P.B., Choi, E.Y., Finberg, K., Roeder, G.S., and Snyder, M. (1994). Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. Genes Dev. *8*, 1087–1105.

Carvajal, E., van den Hazel, H.B., Cybularz-Kolaczkowska, A., Balzi, E., and Goffeau, A. (1997). Molecular and phenotypic characterization of yeast *PDR1* mutants that show hyperactive transcription of various ABC multidrug transporter genes. Mol. Gen. Genet. *256*, 406–415.

Chen, C.Y., Ingram, M.F., Rosal, P.H., and Graham, T.R. (1999). Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. J. Cell Biol. *147*, 1223–1236.

Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. Gene *110*, 119–122.

Coste, H., Martel, M.B., and Got, R. (1986). Topology of glucosylceramide synthesis in Golgi membranes from porcine submaxillary glands. Biochim. Biophys. Acta *858*, 6–12.

Decottignies, A., Grant, A.M., Nichols, J.W., de Wet, H., McIntosh, D.B., and Goffeau, A. (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J. Biol. Chem. *273*, 12612–12622.

Dekkers, D.W., Comfurius, P., Schroit, A.J., Bevers, E.M., and Zwaal, R.F. (1998). Transbilayer movement of NBD-labeled phospholipids in red blood cell membranes: outward-directed transport by the multidrug resistance protein 1 (MRP1). Biochemistry *37*, 14833–14837.

DeRisi, J., van den Hazel, B., Marc, P., Balzi, E., Brown, P., Jacq, C., and Goffeau, A. (2000). Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. FEBS Lett. *470*, 156–160.

Funato, K., Lombardi, R., Vallee, B., and Riezman, H. (2003). Lcb4p is a key regulator of ceramide synthesis from exogenous long chain sphingoid base in *Saccharomyces cerevisiae*. J. Biol. Chem. *278*, 7325–7334.

Futerman, A.H., and Pagano, R.E. (1991). Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. Biochem. J. *280*, 295–302.

Futerman, A.H., Stieger, B., Hubbard, A.L., and Pagano, R.E. (1990). Sphingomyelin synthesis in rat liver occurs predominantly at the *cis* and *medial* cisternae of the Golgi apparatus. J. Biol. Chem. *265*, 8650–8657.

Hannun, Y.A., Luberto, C., and Argraves, K.M. (2001). Enzymes of sphingolipid metabolism: from modular to integrative signaling. Biochemistry *40*, 4893–4903.

Hanson, P.K., and Nichols, J.W. (2001). Energy-dependent flip of fluorescence-labeled phospholipids is regulated by nutrient starvation and transcription factors, *PDR1* and *PDR3*. J. Biol. Chem. *276*, 9861–9867.

Hanson, P.K., Malone, L., Birchmore, J.L., and Nichols, J.W. (2003). Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. J. Biol. Chem. *278*, 36041–36050.

Hata, H., Mitsui, H., Liu, H., Bai, Y., Denis, C.L., Shimizu, Y., and Sakai, A. (1998). Dhh1p, a putative RNA helicase, associates with the general transcription factors Pop2p and Ccr4p from *Saccharomyces cerevisiae*. Genetics *148*, 571–579.

Hua, Z., Fatheddin, P., and Graham, T.R. (2002). An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. Mol. Biol. Cell *13*, 3162–3177.

Jeckel, D., Karrenbauer, A., Burger, K.N., van Meer, G., and Wieland, F. (1992). Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. J. Cell Biol. *117*, 259–267.

Kato, U., Emoto, K., Fredriksson, C., Nakamura, H., Ohta, A., Kobayashi, T., Murakami-Murofushi, K., and Umeda, M. (2002). A novel membrane protein,

Ros3p, is required for phospholipid translocation across the plasma membrane in *Saccharomyces cerevisiae*. J. Biol. Chem. *277*, 37855–37862.

Katzmann, D.J., Hallstrom, T.C., Mahe, Y., and Moye-Rowley, W.S. (1996). Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene *PDR5*. J. Biol. Chem. *271*, 23049–23054.

Katzmann, D.J., Hallstrom, T.C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Moye-Rowley, W.S. (1995). Expression of an ATP-binding cassette transporter-encoding gene (*YOR1*) is required for oligomycin resistance in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *15*, 6875–6883.

Kean, L.S., Grant, A.M., Angeletti, C., Mahe, Y., Kuchler, K., Fuller, R.S., and Nichols, J.W. (1997). Plasma membrane translocation of fluorescent-labeled phosphatidylethanolamine is controlled by transcription regulators, *PDR1* and *PDR3*. J. Cell Biol. *138*, 255–270.

Kihara, A., and Igarashi, Y. (2002). Identification and characterization of a *Saccharomyces cerevisiae* gene, *RSB1*, involved in sphingoid long-chain base release. J. Biol. Chem. *277*, 30048–30054.

Kihara, A., Sano, T., Iwaki, S., and Igarashi, Y. (2003). Transmembrane topology of sphingoid long-chain base-1-phosphate phosphatase, Lcb3p. Genes Cells *8*, 525–535.

Kim, S., Fyrst, H., and Saba, J. (2000). Accumulation of phosphorylated sphingoid long chain bases results in cell growth inhibition in *Saccharomyces cerevisiae*. Genetics *156*, 1519–1529.

Kornberg, R.D., and McConnell, H.M. (1971). Inside-outside transitions of phospholipids in vesicle membranes. Biochemistry *10*, 1111–1120.

Lannert, H., Bunning, C., Jeckel, D., and Wieland, F.T. (1994). Lactosylceramide is synthesized in the lumen of the Golgi apparatus. FEBS Lett. *342*, 91–96.

Lucau-Danila, A., Delaveau, T., Lelandais, G., Devaux, F., and Jacq, C. (2003). Competitive promoter occupancy by two yeast paralogous transcription factors controlling the multidrug resistance phenomenon. J. Biol. Chem. *278*, 52641–52650.

Maillet, L., and Collart, M.A. (2002). Interaction between Not1p, a component of the Ccr4-Not complex, a global regulator of transcription, and Dhh1p, a putative RNA helicase. J. Biol. Chem. *277*, 2835–2842.

Mandon, E.C., Ehses, I., Rother, J., van Echten, G., and Sandhoff, K. (1992). Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dihydrosphinganine reductase, and sphinganine *N*-acyltransferase in mouse liver. J. Biol. Chem. *267*, 11144–11148.

Mao, C., Saba, J.D., and Obeid, L.M. (1999). The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. Biochem. J. *342*, 667–675.

Marx, U., Polakowski, T., Pomorski, T., Lang, C., Nelson, H., Nelson, N., and Herrmann, A. (1999). Rapid transbilayer movement of fluorescent phospholipid analogues in the plasma membrane of endocytosis-deficient yeast cells does not require the Drs2 protein. Eur. J. Biochem. *263*, 254–263.

Meyers, S., Schauer, W., Balzi, E., Wagner, M., Goffeau, A., and Golin, J. (1992). Interaction of the yeast pleiotropic drug resistance genes *PDR1* and *PDR5*. Curr. Genet *21*, 431–436.

Nourani, A., Papajova, D., Delahodde, A., Jacq, C., and Subik, J. (1997). Clustered amino acid substitutions in the yeast transcription regulator Pdr3p increase pleiotropic drug resistance and identify a new central regulatory domain. Mol. Gen. Genet. *256*, 397–405.

Obeid, L.M., Okamoto, Y., and Mao, C. (2002). Yeast sphingolipids: metabolism and biology. Biochim. Biophys. Acta *1585*, 163–171.

Pomorski, T., Lombardi, R., Riezman, H., Devaux, P.F., van Meer, G., and Holthuis, J.C. (2003). Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol. Biol. Cell *14*, 1240–1254.

Qie, L., Nagiec, M.M., Baltisberger, J.A., Lester, R.L., and Dickson, R.C. (1997). Identification of a *Saccharomyces cerevisiae* gene, *LCB3*, necessary for incorporation of exogenous long chain base into sphingolipids. J. Biol. Chem. *272*, 16110–16117.

Robinson, J.S., Klionsky, D.J., Banta, L.M., and Emr, S.D. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery an processing of multiple vacuolar hydrolases. Mol. Cell. Biol. *8*, 4936–4948.

Rogers, B., Decottignies, A., Kolaczkowski, M., Carvajal, E., Balzi, E., and Goffeau, A. (2001). The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*. J. Mol. Microbiol. Biotechnol. *3*, 207–214.

Saba, J.D., Nara, F., Bielawska, A., Garrett, S., and Hannun, Y.A. (1997). The *BST1* gene of *Saccharomyces cerevisiae* is the sphingosine-1-phosphate lyase. J. Biol. Chem. *272*, 26087–26090.

Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004). Cdc50p, a protein required for polarized growth, associates with the Drs2p P-type ATPase implicated in phospholipid translocation in *Saccharomyces cerevisiae*. Mol. Biol. Cell *15*, 3418–3432.

Schroit, A.J., and Zwaal, R.F. (1991). Transbilayer movement of phospholipids in red cell and platelet membranes. Biochim. Biophys. Acta *1071*, 313–329.

Siegmund, A., Grant, A., Angeletti, C., Malone, L., Nichols, J.W., and Rudolph, H.K. (1998). Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 34399–34405.

Smit, J.J., *et al*. (1993). Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell *75*, 451–462.

Smith, A.J., Timmermans-Hereijgers, J.L., Roelofsen, B., Wirtz, K.W., van Blitterswijk, W.J., Smit, J.J., Schinkel, A.H., and Borst, P. (1994). The human MDR3 P-glycoprotein promotes translocation of phosphatidylcholine through the plasma membrane of fibroblasts from transgenic mice. FEBS Lett. *354*, 263–266.

Spiegel, S., and Milstien, S. (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat. Rev. Mol. Cell. Biol. *4*, 397–407.

Sprong, H., Kruithof, B., Leijendekker, R., Slot, J.W., van Meer, G., and van der Sluijs, P. (1998). UDP-galactose:ceramide galactosyltransferase is a class I integral membrane protein of the endoplasmic reticulum. J. Biol. Chem. *273*, 25880–25888.

Tang, X., Halleck, M.S., Schlegel, R.A., and Williamson, P. (1996). A subfamily of P-type ATPases with aminophospholipid transporting activity. Science *272*, 1495–1497.

van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., and van Meer, G. (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell *87*, 507–517.

Williamson, P., and Schlegel, R.A. (2002). Transbilayer phospholipid movement and the clearance of apoptotic cells. Biochim. Biophys. Acta *1585*, 53–63.

Zanolari, B., Friant, S., Funato, K., Sutterlin, C., Stevenson, B.J., and Riezman, H. (2000). Sphingoid base synthesis requirement for endocytosis in *Saccharomyces cerevisiae*. EMBO J. *19*, 2824–2833.

Zhang, X., Skrzypek, M.S., Lester, R.L., and Dickson, R.C. (2001). Elevation of endogenous sphingolipid long-chain base phosphates kills *Saccharomyces cerevisiae* cells. Curr. Genet. *40*, 221–233.