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The Rim101 Pathway Is Involved in Rsb1 Expression Induced by Altered Lipid Asymmetry

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Biological membranes consist of lipid bilayers. The lipid compositions between the two leaflets of the plasma membrane differ, generating lipid asymmetry. Maintenance of proper lipid asymmetry is physiologically quite important, and its collapse induces several cellular responses including apoptosis and platelet coagulation. Thus, a change in lipid asymmetry must be restored to maintain "lipid asymmetry homeostasis." However, to date no lipid asymmetry-sensing proteins or any related downstream signaling pathways have been identified. We recently demonstrated that expression of the putative yeast sphingoid long-chain base transporter/translocase Rsb1 is induced when glycerophospholipid asymmetry is altered. Using mutant screening, we determined that the pH-responsive Rim101 pathway, the protein kinase Mck1, and the transcription factor Mot3 all act in lipid asymmetry signaling, and that the Rim101 pathway was activated in response to a change in lipid asymmetry. The activated transcription factor Rim101 induces Rsb1 expression via repression of another transcription repressor, Nrg1. Changes in lipid asymmetry are accompanied by cell surface exposure of negatively charged phospholipids; we speculate that the Rim101 pathway recognizes the surface charges.

INTRODUCTION

Biological membranes are composed of lipid bilayers, and in the plasma membrane the lipid compositions differ between the two leaflets, resulting in asymmetry. Glycerophospholipids and sphingolipids contribute greatly to such asymmetry. Phosphatidylcholine (PC) and complex sphingolipids, including sphingomyelin (SM) and glycosphingolipids in mammals and *myo*-inositol–containing sphingolipids in the yeast *Saccharomyces cerevisiae*, are located mainly in the outer (extracytosolic) leaflet. Conversely, phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are confined to the inner (cytosolic) leaflet (Pomorski *et al.*, 2004; Ikeda *et al.*, 2006). The hydrophilic nature of the headgroups of these amphiphilic lipids hinders their ability to traverse the hydrophobic membrane interior, and spontaneous flip-flop of the protein-free model membrane occurs only in low frequency (Bai and Pagano, 1997). However, situated in the biological membranes are enzymes called lipid translocases or flippases, which catalyze the transbilayer movement of lipids and establish their asymmetry. Recent studies have identified three classes of translocases/transporters for glycerophospholipids: ABC transporters, P-type ATPases, and scramblases (Holthuis and Levine, 2005; Ikeda *et al.*, 2006). ABC transporters catalyze flop, which is the movement from the cytosolic to extracytosolic leaflet, whereas P-type ATPases stimulate flip, the reverse movement. Scramblases randomize the lipid distribution between the two leaflets. Sometimes, discriminating between a translocase and transporter is difficult. For example, it is unclear whether a lipid in one leaflet is directly translocated to the other leaflet or is first transported to the other side of the hydrophilic fluid and then reincorporated into the membrane.

Maintenance of proper lipid asymmetry is important for several membrane functions. For instance, in mammals skeletal proteins like spectrin improve the mechanical stability of red blood cells by interacting with PS in the inner leaflet (Manno *et al.*, 2002). Similarly, when certain glycerophospholipid translocase genes (*DNF1*, *DNF2*, *DNF3*, and *DRS2*) are deleted in yeast, intracellular trafficking and maintenance of organelle structure are impaired (Chen *et al.*, 1999; Gall *et al.*, 2002; Hua *et al.*, 2002; Pomorski *et al.*, 2003; Natarajan *et al.*, 2004; Saito *et al.*, 2004; Furuta *et al.*, 2007). On the other hand, local or global changes in lipid asymmetry can induce several cellular responses. PS exposed on the outer leaflet of apoptotic cells, as a result of membrane collapse, is used as a recognition signal by phagocytes (Fadok *et al.*, 1992). PS exposure on activated platelets is also essential for blood coagulation (Zwaal *et al.*, 1998; Lentz, 2003). In addition, transient PE exposure and loss of cell surface SM have been observed at cleavage furrows during cytokinesis, and the interaction of exposed PE with PE-binding compounds results in cell cycle arrest (Emoto *et al.*, 1996).

Some, but not all, ABC transporters catalyze the flop of glycerophospholipids (Ikeda *et al.*, 2006). In mammals, translocation/transport of glycerophospholipids (with little selectivity) by ABCB1 (MDR1) has been reported (van Helvoort *et al.*, 1996), as has that of PC by ABCB4 (human

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Abbreviations used: Endo H, endoglycosidase H; ESCRT, endosomal sorting complex required for transport; HA, hemagglutinin; NRE, Nrg1-responsive element; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgk1, phosphoglycerokinase 1; PI, phosphatidylinositol; PS, phosphatidylserine; Ro, Ro 09-0198; SC, synthetic complete; SM, sphingomyelin.

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MDR3, mouse mdr2; Smit *et al.,* 1993), PS by ABCA1 (Abramova *et al.,* 2001), and *N*-retinylidene-PE by ABCA4 (Weng *et al.,* 1999). In yeast, Pdr5 and Yor1 are thought to be involved in the flop of glycerophospholipids (Decottignies *et al.,* 1998; Pomorski *et al.,* 2003). *PDR5* and *YOR1* are regulated by the transcription factor Pdr1, and their up-regulation in a gain-of-function *PDR1-3* mutant causes a reduction in the accumulation of labeled PE, probably due to increased efflux (Decottignies *et al.,* 1998). A Pdr5/Yor1-dependent increase in endogenous PE on the cell surface was later confirmed (Pomorski *et al.,* 2003).

A subfamily of P-type ATPases (type 4 subfamily or aminophospholipid translocases) mediates the flip of glycerophospholipids. In mammals, ATP8A1, ATP8B1, and ATP8B3 are reportedly involved in PS translocation (Ujhazy *et al.,* 2001; Wang *et al.,* 2004; Paterson *et al.,* 2006). In yeast, five proteins belong to this family, Dnf1 and Dnf2 in the plasma membrane and Neo1, Drs2, and Dnf3, which reside in the internal membranes (Pomorski *et al.,* 2003). Disruption of the *DNF1* and *DNF2* genes abolishes the ATP-dependent flip of fluorescent-labeled PC, PE, and PS as well as any reduction in cell surface PE, indicating that Dnf1 and Dnf2 have important roles in maintaining the glycerophospholipid asymmetry of the plasma membrane (Pomorski *et al.,* 2003). To properly target the plasma membrane, Dnf1 and Dnf2 require association with a common subunit, Lem3 (also known as Ros3), a member of the Cdc50 family (Saito *et al.,* 2004; Furuta *et al.,* 2007). Therefore, a *lem3*Δ mutant exhibits effects similar to those of a *dnf1*Δ *dnf2*Δ double mutant, i.e., defective accumulation of exogenously added fluorescent-labeled PC or PE (Kato *et al.,* 2002; Hanson *et al.,* 2003).

Although sphingolipids also contribute to lipid asymmetry formation, knowledge of sphingolipid translocases is limited. In yeast, we identified Rsb1 (Yor049c) as a putative sphingoid long-chain base-specific translocase/transporter (Kihara and Igarashi, 2002). Rsb1 apparently mediates the flop or efflux of long-chain base in an ATP-dependent manner (Kihara and Igarashi, 2002, 2006). Rsb1 expression is low in wild-type cells under normal growth conditions. However, its expression is significantly induced when glycerophospholipid asymmetry is altered, such as by mutations in genes involved in either the flip (P-type ATPases *DNF1* and *DNF2* or the regulatory subunit *LEM3*) or the flop (ABC transporters *PDR5* and *YOR1*) of glycerophospholipids (Kihara and Igarashi, 2004). Conversely, Rsb1 overproduction promotes the flip and represses the flop of fluorescent-labeled PC and PE (Kihara and Igarashi, 2004). This suggests the existence of cross-talk between glycerophospholipids and sphingolipids in lipid asymmetry formation and of sensor molecules that detect changes in glycerophospholipid asymmetry or in downstream signal transduction pathways leading to Rsb1 expression. However, information regarding such factors and pathways is completely lacking.

To identify factors required for Rsb1 induction, we screened for mutants having defects in the Rsb1 expression normally induced by alternations in lipid asymmetry. This screening identified five genes (*MCK1*, *MOT3*, *RIM13*, *RIM20*, and *RIM21*) in addition to the previously characterized gene *PDR1*. Of these genes, three (*RIM13*, *RIM20*, and *RIM21*) are known to be involved in the alkaline pH-responsive Rim101 pathway. Further analyses revealed that the Rim101 pathway is indispensable for the induction of Rsb1. Thus, our findings provide new insight into signaling associated with lipid asymmetry, which is the convergence of lipid asymmetry and alkaline pH adaptation through the Rim101 pathway.

MATERIALS AND METHODS

Yeast Strains and Media

S. cerevisiae strains used are listed in Table 1. Cells were grown in either YPD medium (1% yeast extract, 2% bactopeptone, and 2% D-glucose) or synthetic complete (SC) medium (0.67% yeast nitrogen base and 2% D-glucose) containing nutritional supplements. Buffered SC was prepared using 0.1 M sodium citrate buffers (pH 4.4 and 5.4) or 0.1 M sodium phosphate buffers (pH 6.4 and 7.4). The tetracyclic peptide antibiotic Ro 09-0198 (cinnamycin), kindly provided by Dr. Masato Umeda (Kyoto University, Japan), was dissolved in dimethyl sulfoxide/water, 1:1 (vol/vol). Ro 09-0198 and the peptide-derived drug proteasome inhibitor MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal; Sigma, St. Louis, MO) were diluted in YPD medium for use in drug sensitivity assays.

The chromosomal *RSB1* gene was tagged at its 3′-terminus with three copies of a hemagglutinin (HA) epitope. This was achieved by replacing the *RSB1* gene with a fragment containing both the *RSB1-HA* and a *TRP1* marker by essentially the same method described previously (Uemura *et al.,* 2007).

Plasmids

Primers and templates used are listed in Tables 2 and 3. The pFI1 plasmid (Hayashi *et al.,* 2005), which encodes N-terminally triple HA-tagged Rim101 (HA-Rim101), was a kind gift from Dr. Tatsuya Maeda (Tokyo University, Japan). The pIKD412 plasmid, which is derived from the pRS423 vector (2 μ, *HIS3* marker) (Christianson *et al.,* 1992), encodes *RSB1-HA* under the control of the *RSB1* promoter (P_{RSB1}). Three putative Nrg1-binding sites in pIKD412 were mutated by site-direct mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA).

The pIKD509 (P_{TDH3}-*Myc-RIM101-531*) plasmid, which encodes a constitutively active form of Rim101, was constructed as follows. The *RIM101* gene was amplified using genomic DNA prepared from SEY6210 cells and the primers RIM101-3 and RIM101-4 (Table 2). The resulting fragment was cloned into a pGEM-T Easy (Promega, Madison, WI) vector to generate the pAD21 plasmid. The 1.9-kb BamHI-NotI region of pAD21 was then cloned into the BamHI-NotI site of pAK303, which is a derivative of the pRS315 vector (*CEN*, *LEU2* marker) (Sikorski and Hieter, 1989) designed to produce an N-terminal Myc-tagged protein under the control of the *TDH3* promoter (P_{TDH3}), generating the pIKD432 plasmid. A stop codon was then inserted between codons 531 and 532 by site-direct mutagenesis using a QuikChange kit (Stratagene) and primers RIM101-5 and RIM101-6, creating pIKD509.

For use in the β-galactosidase (LacZ) assay, the pIKD493 (P_{RSB1}-*lacZ*) plasmid was constructed as follows. The pBgal-Basic plasmid (Clontech, Mountain View, CA), which encodes the *lacZ* gene, was digested with SgrAI, blunted using KOD polymerase (Toyobo, Osaka, Japan), and digested further with XhoI. The resulting fragment was cloned into the XhoI-SmaI site of the pRS423 vector, producing the pIKD491 plasmid. The *RSB1* promoter from the pIKD412 plasmid was then amplified using primers RSB1-33 and RSB1-34 (Table 2), and its XhoI-XhoI fragment was inserted into the XhoI site of the pIKD491 plasmid, creating the pIKD493 plasmid.

Screening for Mutants Defective in Lipid Asymmetry Signaling

To identify genes coding for the presumed lipid asymmetry-sensing factor and/or factors involved in its downstream signaling pathways, we screened for mutants exhibiting defects in change-induced Rsb1 expression. For this purpose, the *RSB1* gene was replaced with *ADE2*, which codes for a protein involved in the biosynthesis of purine nucleotides, although the *RSB1* promoter was left intact. Reduced Ade2 levels cause an accumulation of a red purine precursor, making the colony appear red, and, in this case, reflecting the *RSB1* promoter activity. KCY113 (*pdr5*Δ P_{RSB1}-*ADE2*) cells, used in the mutant screening, were constructed as follows.

The *ADE2* gene was amplified by PCR using genomic DNA prepared from SEY6210 cells as a template and the primers ADE2-3 and ADE2-4 (Table 2). The amplified fragment was cloned into pGEM-T Easy, creating the pIKD248 plasmid. The 0.9-kb HpaI-BclI *HA-RSB1* region of the pAK464 plasmid (Kihara and Igarashi, 2004), which contains P_{RSB1}-*HA-RSB1–3′-UTR*$_{RSB1}$, was then replaced by the 1.7-kb PvuII-BamHI *ADE2* fragment of pIKD248, creating pIKD249. The P_{RSB1}-*ADE2-3′-UTR*$_{RSB1}$ fragment of pIKD249 was then introduced into KCY72 cells. Cells undergoing homologous recombination between P_{RSB1}-*ADE2-3′-UTR*$_{RSB1}$ and *rsb1*Δ::*URA3* were expected to lose the *URA3* marker, so we selected such cells with 50 μg/ml 5-fluoro-orotic acid. One of the selected clones, KCY86 exhibited the proper genotype and were used in this study. KCY113 (*pdr5*Δ P_{RSB1}-*ADE2*) cells were then constructed by introducing the *pdr5*Δ::*URA3* mutation into the KCY86 cells.

Screening of mutants defective in lipid asymmetry signaling was performed using a genomic library (kindly provided by Dr. Michael Snyder, Yale University, New Haven, CT) that had been mutagenized by random insertion of the transposon mTn-*lacZ/LEU2* (Burns *et al.,* 1994). The genomic library was digested with NotI, and the resulting DNA fragments were transformed into KCY113 cells. Pooled transposon-carrying mutants were plated at $\sim 2.0 \times 10^3$ cells per plate on YPD plates. The plates were then incubated at 30°C for 2 d. Mutants exhibiting a darker red than that of KCY113 cells were obtained at a

Table 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*MATα leu2-3, 112 ura3-52 his3Δ200 trp1Δ901 lys2-801 suc2Δ9*	Robinson *et al.* (1988)
KCY72	SEY6210, *rsb1Δ::URA3 ade2Δ::TRP1*	This study
KCY86	SEY6210, *rsb1Δ::P_{RSB1}-ADE2 ade2Δ::TRP1*	This study
KCY113	SEY6210, *rsb1Δ::P_{RSB1}-ADE2 ade2Δ::TRP1 pdr5Δ::URA3*	This study
KCY594	SEY6210, *nrg1Δ::KanMX4*	This study
KCY595	SEY6210, *rim101Δ::KanMX4*	This study
KCY662	SEY6210, *rsb1::RSB1-HA TRP1*	This study
KCY689	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3*	This study
KCY692	SEY6210, *rsb1::RSB1-HA TRP1 lem3Δ::HIS3*	This study
KCY694	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 pdr1Δ::LEU2*	This study
KCY696	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 lem3Δ::HIS3*	This study
KCY697	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim20Δ::KanMX4*	This study
KCY1011	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 yor1Δ::KanMX4*	This study
KCY1012	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 mck1Δ::KanMX4*	This study
KCY1013	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 mot3Δ::KanMX4*	This study
KCY1014	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim13Δ::KanMX4*	This study
KCY1015	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim21Δ::KanMX4*	This study
KCY1016	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim101Δ::KanMX4*	This study
KCY1029	SEY6210, *rsb1::RSB1-HA TRP1 dnf1Δ::KanMX4 dnf2Δ::URA3*	This study
KCY1046	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 dfg16Δ::KanMX4*	This study
KCY1047	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim9Δ::KanMX4*	This study
KCY1048	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim8Δ::KanMX4*	This study
KCY1049	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 ygr122wΔ::KanMX4*	This study
KCY1051	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 vps28Δ::KanMX4*	This study
KCY1052	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 vps25Δ::KanMX4*	This study
KCY1053	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 snf7Δ::KanMX4*	This study
KCY1054	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 vps20Δ::KanMX4*	This study
KCY1055	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 did4Δ::KanMX4*	This study
KCY1056	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 vps24Δ::KanMX4*	This study
KCY1060	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 ime1Δ::KanMX4*	This study
KCY1061	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 nrg1Δ::KanMX4*	This study
KCY1062	SEY6210, *rsb1::RSB1-HA TRP1 lem3Δ::HIS3 mck1Δ::KanMX4*	This study
KCY1063	SEY6210, *rsb1::RSB1-HA TRP1 lem3Δ::HIS3 mot3Δ::KanMX4*	This study
KCY1064	SEY6210, *rsb1::RSB1-HA TRP1 lem3Δ::HIS3 rim101Δ::KanMX4*	This study
KCY1065	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 mot3Δ::KanMX4 mck1Δ::LEU2*	This study
KCY1066	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 mot3Δ::KanMX4 rim101Δ::LEU2*	This study
KCY1067	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 rim101Δ::KanMX mck1Δ::LEU2*	This study
KCY1070	SEY6210, *rsb1::RSB1-HA TRP1 pdr5Δ::URA3 nrg1Δ::KanMX4 rim101Δ::LEU2*	This study
KCY1102	SEY6210, *rsb1::RSB1-HA TRP1 mck1Δ::KanMX4*	This study
KCY1103	SEY6210, *rsb1::RSB1-HA TRP1 mot3Δ::KanMX4*	This study
KCY1104	SEY6210, *rsb1::RSB1-HA TRP1 rim101Δ::KanMX4*	This study
KCY1112	SEY6210, *rsb1::RSB1-HA TRP1 URA3*	This study
KCY1141	SEY6210, *rsb1::RSB1-HA TRP1 lem3Δ::KanMX4 URA3*	This study

frequency of ~1/1400. Of these, we chose 15 mutants for further analysis. The sites of transposon insertion in the isolated mutants were determined according to the manuals of the Yale Genome Analysis Center (http://ygac.med.yale.edu/).

Immunoblotting

Yeast cells were precultured overnight in YPD medium at 30°C and then diluted into YPD medium to 0.3 OD_{600} unit/ml. Cells bearing plasmids were precultured in SC medium instead of YPD medium. After being grown at 30°C to ~1 OD_{600} unit/ml, the cells were collected. Preparation of total cell lysate and immunoblotting were performed as described previously (Kihara and Igarashi, 2002). Protein concentrations were measured using a BCA protein assay reagent (Pierce, Rockford, IL). Immunoblotting was performed using as primary antibodies anti-HA (Y-11; 0.16 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Myc (PL-14; 1 μg/ml; Medical and Biological Laboratories, Nagoya, Japan), and anti-phosphoglycerokinase 1 (Pgk1; 22C5; 0.0625 μg/ml; Molecular Probes, Eugene, OR) antibodies. HRP-conjugated anti-rabbit or anti-mouse IgG $F(ab')_2$ (each from GE Healthcare Bio-Sciences, Piscataway, NJ; diluted 1:10,000) was used as the secondary antibody. Labeling was detected using an enhanced chemiluminescence (ECL) or ECL plus kit (GE Healthcare Bio-Sciences). For detecting HA-Rim101, Myc-Rim101-531, and genomic-encoded Rsb1-HA proteins, we enhanced the signal of the anti-HA antibody Y-11 and the anti-Myc antibody PL-14 using Can Get Signal Immunoreaction Enhancer Solution (Toyobo), according to the manufacturer's manual.

Deglycosylation

Proteins (5 μl) in 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and a trace amount of bromophenol blue containing 5% 2-mercaptoethanol) were diluted with 40 μl 0.05 M sodium citrate (pH 5.5) containing 1 mM phenylmethylsulfonyl fluoride, and treated at 37°C for 1 h with 1000 U of recombinant endoglycosidase H (Endo H; New England Biolabs, Beverly, MA). The samples were then mixed with 12 μl 4× SDS sample buffer and 3 μl 2-mercaptoethanol and incubated at 37°C for 5 min. A portion of each sample was separated by SDS-PAGE and subjected to immunoblotting.

β-Galactosidase Assay

β-Galactosidase activities were determined as described elsewhere (Simon and Lis, 1987), with minor modifications. Cells bearing the pIKD493 plasmid (P_{RSB1}-*lacZ*) were precultured in SC medium lacking histidine then diluted into 5 ml YPD medium. After a 4-h incubation at 30°C, the cells were washed with 1 ml buffer I (10 mM Tris-HCl, pH 7.5, and 5 mM dithiothreitol) and suspended in 50 μl buffer I. Acid-washed glass beads (Sigma) were added to the cells, and the cells were broken by mixing vigorously for 15 min at 4°C. Another 50 μl of buffer I was then added to the cells, and the samples were mixed vigorously for 5 min. Cell debris was removed by centrifugation, and the supernatants were subjected to a β-galactosidase assay. Before initiating the reaction, 144 μl assay buffer (50 mM potassium phosphate, pH 7.5, and 1 mM $MgCl_2$) and 3 μl of 50 mM chlorophenol red-β-D-galactopyranoside (the substrate) were mixed and

Table 2. Primers used in this study

Primer	Nucleotide sequence
ADE2-3	5′-CAGCTGATGGATTCTAGAACAGTTGGTATATTAG-3′ (PvuII)
ADE2-4	5′-GGATCCTTACTTGTTTTCTAGATAAGCTTCGTAAC-3′ (BamHI)
RIM101-3	5′-GGATCCGTGCCATTGGAAGATCTGCTTAAT-3′ (BamHI)
RIM101-4	5′-TCATACCAAAATTTTGGGATACTTGG-3′
RIM101-5	5′-CTGGGAATTTAGCCTGAACTAGTCAAAAAAGCTGTACTAATG-3′
RIM101-6	5′-CATTAGTACAGCTTTTTTGACTAGTTCAGGCTAAATTCCCAG-3′
RSB1-15	5′-AACAAAACTCTCCGAGATTTCAATCCAGAGCATAG-3′ (mt1)
RSB1-16	5′-CTATGCTCTGGATTGAAATCTCGGAGAGTTTTGTT-3′ (mt1)
RSB1-17	5′-CACGGCCATGCCTTGATTTTTCTGTCGCCCCCTAG-3′ (mt2)
RSB1-18	5′-CTAGGGGGCGACAGAAAAATCAAGGCATGGCCGTG-3′ (mt2)
RSB1-19	5′-TGAGGGTTCTGTCGCAAACTAGCTGTACGTAAGC-3′ (mt3)
RSB1-20	5′-GCTTACGTACAGCTAGTTTGCGACAGAACCCTCA-3′ (mt3)
RSB1-21	5′-TGATTTTTCTGTCGCAAACTAGCTGTACGTAAGC-3′ (mt3)
RSB1-22	5′-GCTTACGTACAGCTAGTTTGCGACAGAAAAATCA-3′ (mt3)
RSB1-33	5′-ATACGACTCACTATAGGGCGAATTGG-3′
RSB1-34	5′-TGCGTTCTCGAGTTTGAATTTCTCAACGTCTATAA-3′ (XhoI)

Underlined letters are the restriction sites created or the mutated nucleotides.

Table 3. Primers and templates used for construction of NRE-mutated plasmids

Plasmid	Primers	Template
pIKD414 (P_{RSB1}*(mt1)-RSB1-HA*)	RSB1-15 and RSB1-16	pIKD412
pIKD416 (P_{RSB1}*(mt2)-RSB1-HA*)	RSB1-17 and RSB1-18	pIKD412
pIKD418 (P_{RSB1}*(mt3)-RSB1-HA*)	RSB1-19 and RSB1-20	pIKD412
pIKD420 (P_{RSB1}*(mt2/3)-RSB1-HA*)	RSB1-21 and RSB1-22	pIKD416

incubated at 37°C. The reaction was started by adding 3 μl cell lysate to the above substrate solution. After a 6-min incubation at 37°C, the reaction was terminated by adding 250 μl 1 M Na_2CO_3, and the β-galactosidase activity was examined by measuring the OD_{574}. The protein concentrations of the cell lysates were measured using a Coomassie (Bradford) protein assay reagent (Pierce). β-Galactosidase activity was normalized to the protein concentration, and the activity of KCY662 (wild-type) cells harboring the pRS423 vector was subtracted as a background. One unit of β-galactosidase was defined as the amount needed per minute to degrade 1 mmol of the substrate chlorophenol red-β-D-galactopyranoside to chlorophenol red and D-galactose.

RESULTS

Accumulation of PE in the Outer Leaflet of pdr5Δ and pdr5Δ yor1Δ Mutants

We previously demonstrated that Rsb1 expression is induced in yeast cells carrying the floppase mutation *pdr5Δ* and is further enhanced with the *pdr5Δ yor1Δ* double mutation (Kihara and Igarashi, 2004). Because up-regulation of Pdr5 and Yor1 in a gain-of-function *PDR1-3* mutant results in a reduction in the amount of PE exposed on the cell surface (Decottignies *et al.*, 1998), we considered that altered lipid asymmetry resulting from the *pdr5Δ* and *pdr5Δ yor1Δ* mutations induced the Rsb1 expression. However, to date the effects of the *pdr5Δ* and *yor1Δ* mutations have not been examined in a wild-type background (*PDR1*$^+$) or in our strain background. Therefore, we investigated the amount of surface-exposed PE in our *pdr5Δ* and *pdr5Δ yor1Δ* cells using the tetracyclic peptide antibiotic Ro 09-0198 (Ro), which specifically binds to PE (Wakamatsu *et al.*, 1990; Umeda and Emoto, 1999). Ro binding to PE results in cytolysis (Aoki *et al.*, 1994; Kato *et al.*, 2002), so the amount of surface-exposed PE can easily be estimated by measuring the overall sensitivity of the cell to Ro. As shown in Figure 1A, treatment of wild-type cells with 50 μM Ro caused a reduction in their growth rate (to ~30%). As expected, *pdr5Δ* cells were more resistant to Ro than were wild-type cells, exhibiting a reduced rate of only 40%. Moreover, introduction of the *yor1Δ* mutation into the *pdr5Δ* cells further enhanced the Ro tolerance. This tolerance may be underestimated, though, because the *pdr5Δ* and *pdr5Δ yor1Δ* mutants tend to accumulate several drugs intracellularly unlike wild-type cells, probably because of diminished ability to pump them out (Leonard *et al.*, 1994; Máhe *et al.*, 1996; Decottignies *et al.*, 1998). Indeed, the

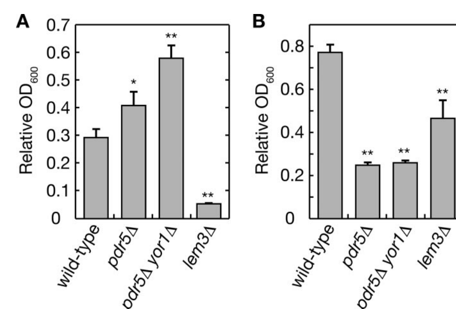

Figure 1. Cell surface PE is reduced in cells carrying the *pdr5Δ* mutation. KCY1112 (wild-type), KCY689 (*pdr5Δ*), KCY1011 (*yor1Δ pdr5Δ*), and KCY1141 (*lem3Δ*) cells were diluted to 0.05 OD_{600} unit/ml in YPD medium containing 50 μM Ro (A) or 200 μM MG132 (B). After a 9-h incubation, the OD_{600} of each culture was measured. Values indicate the OD_{600} for each cell line in the presence of the drugs, relative to that in the absence of the drugs. Values represent the means ± SD from three independent experiments. The statistical significance of each difference as compared with results from wild-type cells was determined using a two-tailed Student's *t* test. *$p < 0.05$; **$p < 0.01$.

*pdr5*Δ and *pdr5*Δ *yor1*Δ mutants were more sensitive to another peptide-derived drug, the proteasome inhibitor MG132, than were wild-type cells (Figure 1B), consistent with results reported by others (Fleming *et al.*, 2002).

In contrast to these floppase mutants, cells carrying the flippase mutant *lem3*Δ were highly sensitive to Ro (Figure 1A), agreeing with a previous report (Kato *et al.*, 2002). The *lem3*Δ mutant was also more sensitive to MG132 than were wild-type cells, suggesting that increased permeability of the cell membrane may contribute in part to the sensitivity, in addition to increased PE exposure. These results confirm that the amount of surface-exposed PE is lower in *pdr5*Δ *yor1*Δ, and *pdr5*Δ cells, respectively, than in wild-type cells.

Isolation of Mutants Defective in Rsb1 Expression Induced by Changes in Lipid Asymmetry

To identify genes coding for the presumed lipid asymmetry-sensing factor and/or factors involved in its downstream signaling pathways, we screened for mTn-*lacZ/LEU2* transposon-inserted mutants unable to activate *RSB1* promoter-dependent expression in response to a mutation in the *PDR5* gene. This screening identified five genes (*MCK1, MOT3, RIM13, RIM20*, and *RIM21*) in addition to the previously characterized gene *PDR1* (Kihara and Igarashi, 2004). Of these genes, three (*RIM13, RIM20*, and *RIM21*) are known to be involved in the pH-responsive Rim101 pathway (Peñalva and Arst, 2004). *MOT3* is a transcription factor gene involved in the repression of anaerobic condition–induced genes (Abramova *et al.*, 2001; Hongay *et al.*, 2002) and *MCK1* encodes a serine/threonine/tyrosine kinase, which shares similarity with kinases of the mammalian glycogen synthase kinase 3 subfamily (Lim *et al.*, 1993).

To confirm that the isolated transposon mutations were indeed responsible for the Rsb1 induction, we introduced a deletion mutation of each of the isolated genes into KCY689 (*pdr5*Δ *RSB1-HA*) cells, in which the chromosomal *RSB1* gene has been C-terminally triple HA-tagged (*RSB1-HA*). Rsb1 is an *N*-glycosylated protein (Panwar and Moye-Rowley, 2006) and as such was detected in immunoblots as a broad band of 57-90 kDa (Figure 2A), which was shifted to a single 41-kDa band upon treatment with Endo H (Figure 2B). Regardless of the level of glycosylation, *pdr5*Δ-induced Rsb1-HA protein expression was indeed reduced by all the mutations tested (Figure 2, A and B). The *pdr1*Δ mutation had the most prominent effect. The *mck1*Δ, *rim13*Δ, *rim20*Δ, and *rim21*Δ mutations caused reduced expression to a similar extent (Figure 2, A and B).

Because our screening method was designed to identify genes involved in the regulation of transcription from the *RSB1* promoter, the isolated mutations must affect Rsb1 expression at the transcriptional level rather than at the posttranslational level, such as by enhanced protein degradation. To confirm this, we performed a β-galactosidase (LacZ) assay using cells in which the expression of LacZ was under the control of the *RSB1* promoter. Consistent with the above result, all the mutations tested repressed *pdr5*Δ-induced β-galactosidase activity. Again the *pdr1*Δ mutation exhibited the most pronounced effect (Figure 2C).

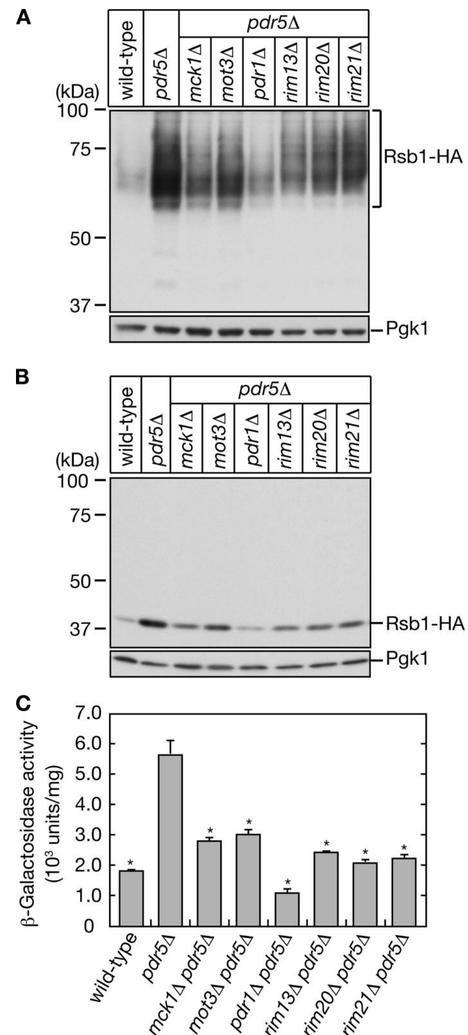

Figure 2. *MCK1*, *MOT3*, *RIM13*, *RIM20*, and *RIM21* are involved in Rsb1 expression induced by changes in lipid asymmetry. KCY662 (wild-type), KCY689 (*pdr5*Δ), KCY1012 (*mck1*Δ *pdr5*Δ), KCY1013 (*mot3*Δ *pdr5*Δ), KCY694 (*pdr1*Δ *pdr5*Δ), KCY1014 (*rim13*Δ *pdr5*Δ), KCY697 (*rim20*Δ *pdr5*Δ), and KCY1015 (*rim21*Δ *pdr5*Δ) cells were grown in YPD medium. (A and B) Total proteins were prepared from each culture. (A) Proteins (13 μg) were separated by SDS-PAGE and then subjected to immunoblotting with an anti-HA antibody or, to demonstrate uniform protein loading, an anti-Pgk1 antibody. (B) Proteins (1.7 μg) were treated with Endo H, separated by SDS-PAGE, and subjected to immunoblotting as in A. (C) Total cell lysates were prepared from each culture and subjected to β-galactosidase reporter assays. Values indicate the means ±SD from three independent experiments. The statistical significance of each difference as compared with results from *pdr5*Δ cells was determined using a two-tailed Student's *t* test. *$p < 0.01$.

The Rim101 Pathway Is Involved in the Induction of Rsb1

In the pH-responsive pathway, Rim13, Rim20, and Rim21 are all located upstream of the transcription factor Rim101 (Hayashi *et al.*, 2005; Boysen and Mitchell, 2006). Rim21 and Dfg16, both multimembrane-spanning proteins, and their homologues are thought to serve as pH sensors (Peñalva and Arst, 2004; Barwell *et al.*, 2005; Herranz *et al.*, 2005; Boysen and Mitchell, 2006). An alkaline signal activates Rim101 via proteolytic processing of its C-terminus by the calpain-like cysteine protease Rim13, with assistance from Rim20 (Li and Mitchell, 1997; Futai *et al.*, 1999; Xu and Mitchell, 2001). Truncated Rim101 then enters the nucleus and modulates the expressions of pH-responsive genes. To investigate whether Rim101 is involved in the induction of Rsb1, a *rim101*Δ mutation was introduced into KCY689 (*pdr5*Δ P_{RSB1}-*RSB1-HA*) cells. The *rim101*Δ mutation reduced the Rsb1-HA expression to a similar extent as the *rim21*Δ, *rim20*Δ, and *rim13*Δ mutations (Figure 3A). This suggests that Rim21, Rim20, and Rim13 regulate the Rsb1 expression via Rim101.

Wild-type cells express weak but detectable amounts of Rsb1, suggesting that local or transient changes in asymmetry constantly occur. We investigated the effects of *mot3*Δ, *mck1*Δ, and *rim101*Δ mutations on Rsb1 expression in the wild-type background. All these mutations caused decreased expression of Rsb1-HA in the wild-type background (Figure 3B) just as they had in the *pdr5*Δ background (Figures 2 and 3A).

Lipid asymmetry is maintained both by ABC transporter–mediated flop and by P-type ATPase-mediated flip, and a mutation in either the transporter (flop mutation; *pdr5*Δ or *pdr5*Δ *yor1*Δ) or the ATPase (flip mutation; *lem3*Δ or *dnf1*Δ *dnf2*Δ) leads to altered lipid asymmetry and induction of Rsb1 (Kihara and Igarashi, 2004). To investigate whether Rsb1 induction caused by a flip mutation would be affected by a *mot3*Δ, *mck1*Δ, or *rim101*Δ mutation, we introduced these mutations into KCY692 (*lem3*Δ P_{RSB1}-*RSB1-HA*) cells. We found that all these mutations also caused reduced Rsb1-HA expression (Figure 3B). Thus, Mot3, Mck1, and the Rim101 pathway are required for the lipid asymmetry signal caused by either flip (Figure 3B) or flop mutations (Figures 2 and 3A).

Several other factors are also known to be involved in the Rim101 pathway. These include Rim8, Rim9, Dfg16, and Ygr122w, as well as certain ESCRT (endosomal sorting complex required for transport) proteins (Li and Mitchell, 1997; Xu *et al.*, 2004; Barwell *et al.*, 2005; Rothfels *et al.*, 2005), which function in sorting membrane proteins to the vacuolar degradation pathway and in multivesicular body formation (Katzmann *et al.*, 2002). To investigate which of these factors might be involved in the *pdr5*Δ-related Rsb1 induction, we introduced mutated versions of each gene into KCY689 (*pdr5*Δ P_{RSB1}-*RSB1-HA*) cells and measured Rsb1-HA expression and β-galactosidase reporter activities. To monitor the activation of Rim101, triple HA-tagged Rim101 (HA-Rim101) was also expressed, and its processing was examined. As shown in Figure 3, C and D, all mutations known to impair Rim101 activation (*rim21*Δ, *dfg16*Δ, *rim9*Δ, *rim8*Δ, *rim13*Δ, *rim20*Δ, *ygr122w*Δ, *vps28*Δ, *vps25*Δ, *snf7*Δ, and *vps20*Δ) caused reduced Rsb1 expression. Mutations in ESCRT factors such as *DID4* and *VPS24* are known to cause constitutive partial activation of Rim101 (Hayashi *et al.*, 2005). We found that these mutations caused a slight induction in Rsb1 expression (Figure 3, C and D). When a constitutive active form of Rim101 (Rim101-531), which contains a deletion at the C-terminous, was overproduced, a marked increase in Rsb1-HA

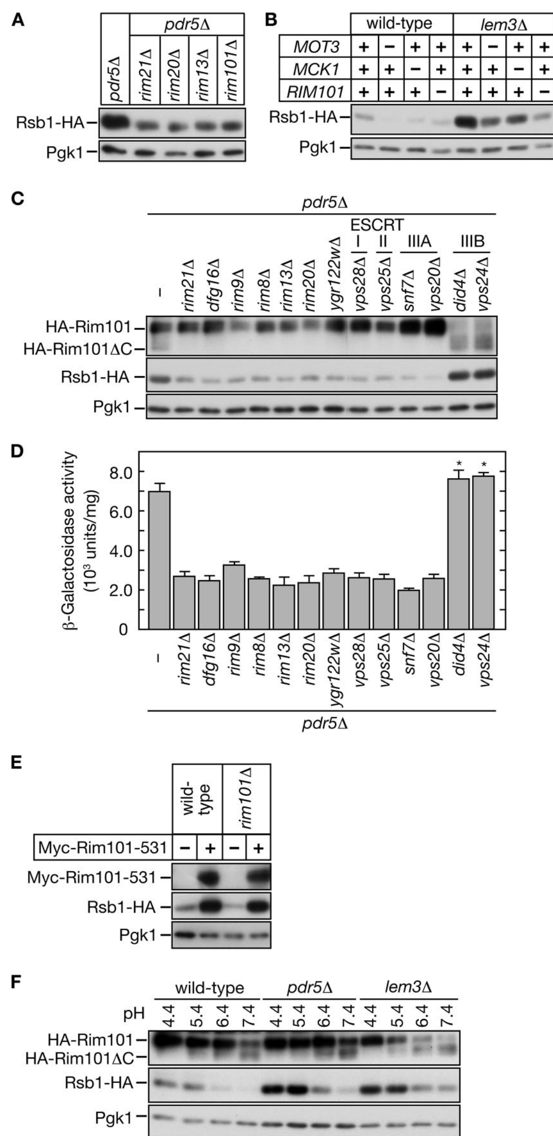

Figure 3. The pH-responsive Rim101 pathway is involved in lipid asymmetry signaling. (A and B) Cells carrying single or double mutations were grown in YPD medium. Total proteins (1.7 μg) prepared from each culture were incubated with Endo H, separated by SDS-PAGE, and subjected to immunoblotting with an anti-HA or anti-Pgk1 antibody. (A) KCY689 (*pdr5*Δ), KCY1014 (*rim13*Δ *pdr5*Δ), KCY697 (*rim20*Δ *pdr5*Δ), KCY1015 (*rim21*Δ *pdr5*Δ), and KCY1016 (*rim101*Δ *pdr5*Δ) cells were used. (B) KCY662 (wild-type), KCY1103 (*mot3*Δ), KCY1102 (*mck1*Δ), KCY1104 (*rim101*Δ), KCY692 (*lem3*Δ), KCY1063 (*mot3*Δ *lem3*Δ), KCY1062 (*mck1*Δ *lem3*Δ), and KCY1064 (*rim101*Δ *lem3*Δ) cells were used. (C and D) KCY689 (*pdr5*Δ), KCY1015 (*rim21*Δ *pdr5*Δ), KCY1046 (*dfg16*Δ *pdr5*Δ), KCY1047 (*rim9*Δ *pdr5*Δ), KCY1048 (*rim8*Δ *pdr5*Δ), KCY1014 (*rim13*Δ *pdr5*Δ), KCY697 (*rim20*Δ *pdr5*Δ), KCY1049 (*ygr122w*Δ *pdr5*Δ), KCY1051 (*vps28*Δ *pdr5*Δ), KCY1052 (*vps25*Δ *pdr5*Δ), KCY1053 (*snf7*Δ *pdr5*Δ), KCY1054 (*vps20*Δ *pdr5*Δ), KCY1055 (*did4*Δ *pdr5*Δ), and KCY1056 (*vps24*Δ *pdr5*Δ) cells bearing the pFI1 (*HA-RIM101*) or pIKD493 (P_{RSB1}-*lacZ*) plasmid were used. (C) Cells harboring the plasmid pFI1 were precultured in SC medium lacking leucine, transferred to YPD medium, and grown to logarithmic phase. Total proteins were prepared from each culture and incubated with Endo H. Proteins (1.25 μg for Rsb1-HA and Pgk1 blots, and 1.7 μg for HA-Rim101 blots) were separated by SDS-PAGE and subject to immunoblotting with an anti-HA or anti-Pgk1 antibody. (D) Cells harboring the plasmid pIKD493 were precultured in SC medium lacking histidine, transferred to YPD medium, and grown to logarithmic phase. Total cell lysates were prepared from each culture and subjected to β-galactosidase reporter assays. Values represent the means ± SD from three independent experiments. The statistical significance of each difference as compared with results from the *pdr5*Δ cells bearing pIKD493 was determined using a two-tailed Student's *t* test. (* $p < 0.05$) (E) KCY662 (wild-type) and KCY1104 (*rim101*Δ) cells harboring the pRS315 or pIKD509 (*Myc-RIM101-531*) plasmid were cultured in SC medium lacking leucine and transferred to YPD medium. Total proteins prepared from each culture were incubated with Endo H. Proteins (2.5 μg for Myc-Rim101-531 blots and 1.25 μg for Rsb1-HA and Pgk1 blots) were separated by SDS-PAGE and subjected to immunoblotting with an anti-Myc, anti-HA, or anti-Pgk1 antibody. (F) KCY662 (wild-type), KCY689 (pdr5Δ), and KCY692 (lem3Δ) cells, each bearing the pFI1 plasmid, were cultured in SC medium lacking leucine for 3 h. An equal volume of buffered SC medium lacking leucine was added to the culture medium, and the cells were incubated for another 2 h. Total proteins were prepared from each culture and incubated with Endo H. Proteins (1.7 μg for HA-Rim101 blots, and 1.25 μg for Rsb1-HA and Pgk1 blots) were separated by SDS-PAGE and subject to immunoblotting with an anti-HA or anti-Pgk1 antibody.

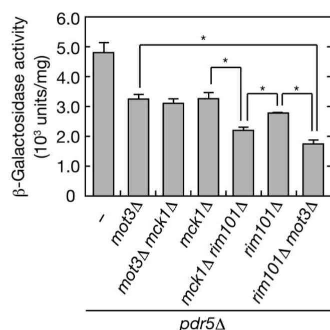

Figure 4. The *rim101*Δ mutation and the *mot3*Δ or *mck1*Δ mutation exhibit additive effects in Rsb1 induction. KCY689 (*pdr5*Δ), KCY1013 (*mot3*Δ *pdr5*Δ), KCY1065 (*mot3*Δ *mck1*Δ *pdr5*Δ), KCY1012 (*mck1*Δ *pdr5*Δ), KCY1067 (*mck1*Δ *rim101*Δ *pdr5*Δ), KCY1016 (*rim101*Δ *pdr5*Δ), and KCY1066 (*rim101*Δ *mot3*Δ *pdr5*Δ) cells harboring the pIKD493 (P_{RSB1}-*lacZ*) plasmid were grown in SC medium lacking histidine. Total cell lysates were prepared from each culture and subjected to β-galactosidase reporter assays. Values represent the means ± SD from three independent experiments. The statistical significance of each difference indicated was determined using a two-tailed Student's *t* test. *$p < 0.01$.

amount was observed both in wild-type and *rim101*Δ cells (Figure 3E).

To further investigate the relationship between the Rim101 pathway and Rsb1 expression induced by changes in lipid asymmetry, we measured the Rsb1-HA in cells exposed to pH levels ranging from 4.4 to 7.4 (Figure 3F). Consistent with previous studies (Hayashi *et al.*, 2005), Rim101 processing was enhanced at higher pH (Figure 3F). In contrast, Rsb1-HA levels were high at low pH (pH 4.4 and 5.4) but low at high pH (pH 6.4 and 7.4; Figure 3F). A similar pH-dependent decrease in Rsb1-HA expression was observed for *pdr5*Δ and *lem3*Δ cells. However, at any pH, the expression of Rsb1-HA was higher in *pdr5*Δ and *lem3*Δ cells than in wild-type cells. Exposure of cells to high pH may induce several cellular responses, any of which might function negatively in the induction of Rsb1 and surpass the effect of the activated Rim101 pathway. For example, as determined by a comprehensive microarray analysis, *PDR1* mRNA is down-regulated at high pH (Causton *et al.*, 2001).

To investigate whether Mot3, Mck1, and the Rim101 pathway regulate Rsb1 expression via the same or different pathways, we generated double mutants for the *MOT3*, *MCK1*, and *RIM101* genes. β-Galactosidase activitiy was additively lower in each double deletion mutant than in the corresponding single deletion mutant, except the activity in the *mot3*Δ *mck1*Δ double mutant, which was similar to that of the *mot3*Δ or *mck1*Δ single mutant (Figure 4). These results suggest that signaling through the Rim101 pathway induces Rsb1 expression independently from the Mot3/Mck1 pathway.

Regulation of the Rim101 Pathway by Changes in Glycerophospholipid Asymmetry

The results described above reveal that the Rim101 pathway is important for the expression of *RSB1*, so we investigated whether changes in lipid asymmetry activate the Rim101 pathway. The amount of cleaved Rim101 was slightly increased in the flop mutants (*pdr5*Δ and *pdr5*Δ *yor1*Δ) compared with wild-type cells, indicating that the Rim101 pathway is activated (Figure 5, A and B). Moreover, we observed more prominent processing of Rim101 in the flip mutants (*lem3*Δ and *dnf1*Δ *dnf2*Δ). Similar results were observed in the pH experiments at all pH levels tested (Figure 3F). Thus,

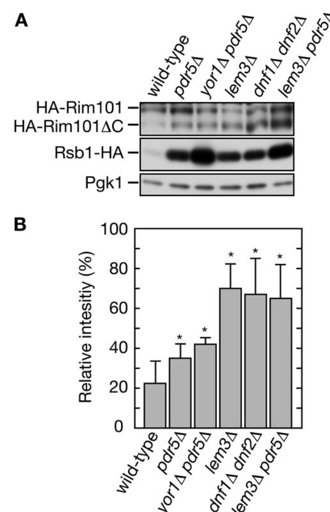

Figure 5. The Rim101 pathway is activated by changes in lipid asymmetry. (A) Total proteins (1.25 μg) prepared from KCY662 (wild-type), KCY689 (*pdr5*Δ), KCY1011 (*yor1*Δ *pdr5*Δ), KCY692 (*lem3*Δ), KCY1029 (*dnf1*Δ *dnf2*Δ), and KCY696 (*lem3*Δ *pdr5*Δ) cells, each harboring the pFI1 (*HA-RIM101*) plasmid, were incubated with Endo H and separated by SDS-PAGE, followed by immunoblotting with an anti-HA or anti-Pgk1 antibody. (B) The intensities of the band for HA-Rim101 and HA-Rim101ΔC presented in A were quantified using Image J software (http://rsb.info.nih.gov/ij/) and are expressed as a percentage reflecting the level of HA-Rim101ΔC relative to sum of the level of the HA-Rim101 plus HA-Rim101ΔC. Values represent the means ± SD from the experiment shown in A and three other independent experiments. The statistical significance of each difference as compared with results from wild-type cells bearing pFI1 was determined using a two-tailed Student's *t* test. *$p < 0.05$.

a change in lipid asymmetry caused by the flip mutations activates the Rim101 pathway more strongly than changes by the flop mutations. Because induction levels of Rsb1-HA are similar between *pdr5*Δ cells and *lem3*Δ or *dnf1*Δ *dnf2*Δ cells, activation of Rim101 cannot be correlated with the Rsb1 induction (Figure 5, A and B). A similar inconsistency was observed between *lem3*Δ cells and *lem3*Δ *pdr5*Δ cells. Although much higher expression of Rsb1-HA was observed in the *lem3*Δ *pdr5*Δ cells compared with the *lem3*Δ cells, similar Rim101 processing was observed (Figure 5, A and B). These results suggest that pathways other than the Rim101 pathway, such as a Mot3/Mck1-related pathway, transduce the lipid asymmetry signal forward, resulting in the expression of Rsb1 in the flop mutants.

Rsb1 Expression Is Repressed Downstream of Rim101 by Nrg1

Because no Rim101 binding sequence exists in the putative promoter region of *RSB1*, it is unlikely that Rim101 directly regulates *RSB1* transcription. Therefore, we searched for candidate transcription factors downstream of Rim101 using the Yeast search for transcriptional regulators and consensus tracking (YEASTRACT) database (http://www.yeastract.com). Of the potential transcription factors identified, two are known to be regulated by Rim101 at the transcriptional level, Ime1 (Su and Mitchell, 1993) and Nrg1 (Lamb and Mitchell, 2003), so we examined whether Rim101 regulates Rsb1 expression via one of these factors. Although an *ime1*Δ mutation had no effect on the *pdr5*Δ-caused Rsb1 expression, an *nrg1*Δ mutation further enhanced the expression (Figure 6A). Moreover, the *nrg1*Δ mutation bypassed the effect of the

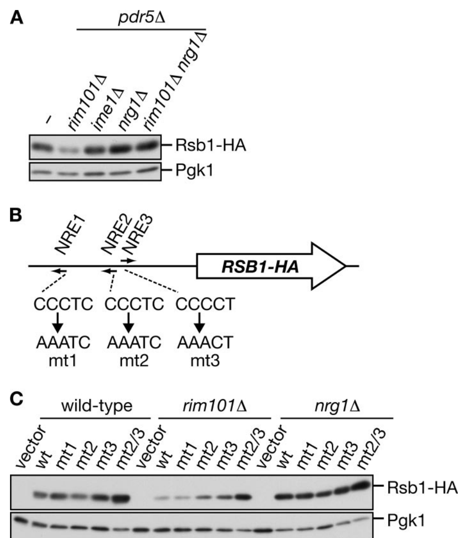

Figure 6. *RSB1* gene expression is repressed by Nrg1, downstream of Rim101. (A) KCY689 (*pdr5*Δ), KCY1016 (*rim101*Δ *pdr5*Δ), KCY1060 (*ime1*Δ *pdr5*Δ), KCY1061 (*nrg1*Δ *pdr5*Δ), and KCY1070 (*rim101*Δ *nrg1*Δ *pdr5*Δ) cells were grown in YPD medium. Total proteins (1.7 μg) prepared from each culture were incubated with Endo H, separated by SDS-PAGE, and subject to immunoblotting with an anti-HA or anti-Pgk1 antibody. (B) Schematic representation of the position and sequences of NREs. The locations of the 5′-nucleotides of NREs are at −761 (NRE1), −470 (NRE2), and −460 (NRE3) relative to the transcription start site. The sequences of the mt1-3 mutations are also shown. (C) The pRS423 (vector), pIKD412 (wild-type; wt), pIKD414 (mt1), pIKD416 (mt2), pIKD418 (mt3), and pIKD420 (mt2/3) plasmids were introduced into SEY6210 (wild-type), KCY595 (*rim101*Δ), and KCY594 (*nrg1*Δ) cells. Cells were precultured in SC medium lacking histidine, transferred to YPD medium, and grown to logarithmic phase. Total proteins (1.25 μg) prepared from each culture were treated with Endo H, separated by SDS-PAGE, and subject to immunoblotting with an anti-HA or anti-Pgk1 antibody.

*rim101*Δ mutation, indicating that Nrg1 functions in Rsb1 induction downstream of Rim101. Rim101 reportedly functions as a repressor for the transcription of Nrg1, which normally represses the expression of pH-inducible genes (Lamb and Mitchell, 2003).

RSB1 has three possible Nrg1-responsive elements (NREs 1-3) in its promoter region (Figure 6B). To determine which element might be important for Nrg1 binding, an *RSB1-HA*-expressing plasmid with its promoter region intact (wt) or carrying one or more mutated NRE sequence (Figure 6B) was introduced into wild-type, *rim101*Δ, and *nrg1*Δ cells. In *nrg1*Δ cells maximal expression occurred whether the promoter region was intact or mutated (Figure 6C), due to the absence of Nrg1, the repressor for *RSB1-HA* mRNA expression. In contrast, Rsb1-HA expression in wild-type cells was slightly increased by the mutation in NRE3 (mt3) and significantly increased by mutations in both NRE2 and NRE3 (mt2/3) compared with the Rsb1 level expressed from the intact promoter. These effects were more evident when the mutated plasmids were introduced into the *rim101*Δ cells (Figure 6C). Expression of Rsb1-HA from the mt2/3 construct in either wild-type cells or *rim101*Δ cells was similar to that observed in the *nrg1*Δ cells. These results suggest that both NRE2 and NRE3 are binding sites for Nrg1.

DISCUSSION

Of five genes identified in this study as coding for the presumed lipid asymmetry-sensing factor or a related protein, three (*RIM13*, *RIM20*, and *RIM21*) are part of the pH-responsive Rim101 pathway. Further analyses revealed that the Rim101 pathway itself is required for Rsb1 induction (Figure 3, C and D). In agreement with this result, a genome-wide microarray analysis found that the *RSB1* gene was down-regulated by a *rim101*Δ or *rim13*Δ mutation (Lamb and Mitchell, 2003). The Rim101 pathway functions in pH adaptation. In *S. cerevisiae*, six *RIM* genes (*RIM8*, *RIM9*, *RIM13*, *RIM20*, *RIM21*, and *RIM101*), *DFG16*, *YGR122w*, and certain ESCRT genes are required for this pathway. The proposed signaling mechanism for this pathway includes several proteins homologous to other known signaling proteins. Dfg16 and Rim21 are homologues to PalH, an *Aspergillus nidulans* protein that interacts with the arrestin homolog PalF (Herranz *et al.*, 2005). Because its multimembrane structure and its interaction with arrestin-like protein are characteristic features of receptor proteins, PalH is considered to be a pH sensor (Peñalva and Arst, 2004; Herranz *et al.*, 2005). By homology, Dfg16 and/or Rim21 are also candidates for being pH sensors. Another multimembrane protein, Rim9, seems to function in association with Dfg16 and Rim21. Dfg16, Rim21, and Rim9 may act as pH sensor subunits or may be required for biogenesis of a sensor protein, e.g., Dfg16 or Rim21. Once Dfg16 or Rim21 recognizes the external alkaline pH, it is subjected to endocytosis by the assistance of the arrestin/PalF homolog Rim8. The adaptor protein Rim20 is then recruited to the endosomal compartment from the cytosol and associates with the ESCRT protein Snf7 (Boysen and Mitchell, 2006), although the molecular mechanism that links Dfg16/Rim21 and Rim20 is unclear. Snf7 seems to also interact with the protease Rim13 (Ito *et al.*, 2001) and to form a multiprotein complex on the endosomal membrane. Rim20 recruits Rim101 to this complex, resulting in the processing of Rim101 by Rim13. The ESCRT-IIIB components are required for dissociation of this complex (Babst *et al.*, 2002), so their mutations cause constitutive activation of Rim101 (Hayashi *et al.*, 2005). The processed Rim101 enters the nucleus and represses the Rim101-responsive genes (Lamb and Mitchell, 2003).

Mutants of type 4 P-type ATPases or Cdc50 family members exhibit not only changes in membrane lipid asymmetry but also in vesicular trafficking functions such as post-Golgi transport and endocytosis (Graham, 2004). The possibility, then, that reduced vesicular transport activity or altered lipid composition indirectly induce Rsb1 expression cannot be excluded. However, none of the mutants affecting post-Golgi transport (*vps45*Δ), endocytosis (*pcl1*Δ and *chc1*Δ), or ergosterol biosynthesis (*erg3*Δ) induced Rsb1 expression (Supplementary Figure 1), suggesting that this possibility is unlikely. Therefore, it is most probable that changes in lipid asymmetry directly induce Rsb1 expression.

At present, it is unclear what the putative sensor proteins Rim21 and/or Dfg16 recognize. Several target molecules can be considered. For example, it is possible that they recognize cell wall components, because the Rim101 pathway is required for normal cell wall assembly (Castrejon *et al.*, 2006). Inconsistent with this possibility, however, is our finding that cell wall mutants (*krt6*Δ and *gas1*Δ) did not induce Rsb1 expression (Supplementary Figure 1). We rather prefer the model, then, that the Rim101 pathway recognizes a cell surface charge. Under normal conditions, the negatively charged phospholipids PI, PS, and phosphatidic acid are confined to the inner leaflet of the plasma membrane. However, changes in lipid asymmetry can expose these negatively charged phospholipids on the cell surface. The putative sensor proteins Rim21 and/or Dfg16 may recognize the exposed negative charge similarly to

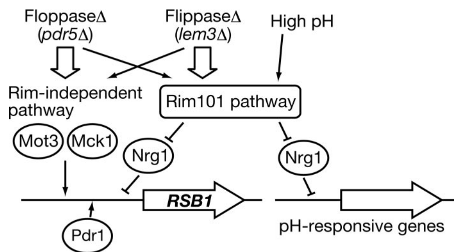

Figure 7. Model of the lipid asymmetry signaling pathway. A signal from moderate increases in pH induces expression of the pH-responsive genes through activation of the Rim101 pathway. The same Rim101 pathway transduces a signal resulting in Rsb1 expression, but other pathways, such as that involving Mot3 and Mck1, are also required. Changes in lipid asymmetry, caused either by a flip or flop mutation, induce activation of the Rim101 pathway and probably of the Mot3/Mck1-related pathways as well. However, these pathways are activated differently by the flip or flop mutation. The Rim101 pathway is activated more strongly by the flip mutation, whereas other pathways seem to be activated more predominantly by the flop mutation. Pdr1 may act as a basic transcription factor, since little Rsb1 expression was observed in *pdr1*Δ cells.

the way they sense a charge in culture medium (hydroxyl ion or proton) under alkaline conditions.

Cooperation between the Rim101 pathway and Mck1 or Mot3 is known in the induction of other genes. For example, both the Rim101 pathway and Mck1 act independently to induce the transcription factor Ime1 (Su and Mitchell, 1993). In addition, the Rim101 pathway and Mot3 are linked via the Tup1-Cyc8 repression complex. Gene repression by Rim101 was shown to be dependent on Tup1-Cyc8 (Park *et al.*, 1999; Lamb and Mitchell, 2003; Rothfels *et al.*, 2005), and Mot3 is reportedly involved in the recruitment of Tup1-Cyc8 to its target sites (Klinkenberg *et al.*, 2005). Therefore, the possibility cannot be excluded that links exist among the Rim101 pathway, Mck1, and Mot3. However, the interpretation that these pathways act independently on Rsb1 induction is more likely considering the additive effects of the *rim101*Δ mutation and the *mck1*Δ or *mot3*Δ mutation (Figure 4). In addition, neither the *mot3*Δ nor *mck1*Δ mutation affected the processing of Rim101 (data not shown).

The lipid asymmetry signals induced by the flip mutation and by the flop mutation do not overlap completely. For example, the flip mutations (*lem3*Δ and *dnf1*Δ *dnf2*Δ) activated the Rim101 pathway more strongly than the flop mutation (*pdr5*Δ), although the Rsb1 induction levels were equivalent (Figure 5). Therefore, in the flop mutant other pathways, such as one involving Mot3/Mck1, must be activated more strongly than the Rim101 pathway (Figure 7).

Although regulation of lipid asymmetry is important for several cellular functions and responses among eukaryotic cells, how changes in lipid asymmetry transduce a signal has been completely unknown. Thus, identification of a role for the Rim101 pathway in this study may provide an important clue for understanding other cellular events governed by lipid asymmetry.

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