S. cerevisiae cells lacking their mitochondrial DNA (ρ0 cells) respond to this loss of genetic information by induction of a program of nuclear gene expression called the retrograde response. Expression of genes involved in multidrug resistance and sphingolipid biosynthesis is coordinately induced in ρ0 cells by the zinc cluster transcription factor Pdr3p. In this report, we identify a membrane protein involved in control of intracellular levels of a sphingolipid precursor as a transcriptional target of the Pdr3p-mediated retrograde response. These sphingolipid precursors are called long chain bases (LCBs) and increased LCB levels are growth inhibitory. This membrane protein has been designated Rsb1p and has previously been shown to act as a LCB transporter protein and to be a component of the endoplasmic reticulum. These earlier studies used an amino-terminal truncated form of Rsb1p. Here we employ a full-length form of Rsb1p and find that this protein is localized to the plasma membrane and is modified by N-linked glycosylation. Two glycosylation sites are present in the Rsb1p and both are required for normal LCB resistance. Mutational analysis of the RSB1 promoter revealed that two Pdr3p binding sites are present and both of these are required for normal retrograde induction of transcription. LCB tolerance is strongly increased in ρ0 cells but this increase is ablated in ρ0 rsb1Δ cells. Together, these data indicate Pdr3p activation of RSB1 transcription is an important feature of the retrograde response allowing normal detoxification of an endogenous sphingolipid precursor.

Sphingolipids are important lipid constituents of eukaryotic membranes. Key intermediates in the biosynthesis of sphingolipids are the long chain bases (LCBs). In S. cerevisiae, these LCBs are dihydrosphingosine and phytosphingosine (PHS) (reviewed in Ref. 1). These LCBs are utilized by ceramide synthase to form ceramide that is a well known bioactive lipid (reviewed in Refs. 2 and 3). However, LCBs and their phosphorylated forms (LCBP) have also recently been found to serve as potent signaling molecules. LCBPs appear to act as inhibitors of proliferation, whereas increased LCBP levels stimulate growth (recently reviewed in Ref. 4). Control of the biological levels of these signaling lipids is an essential feature for ensuring both normal lipid composition of membranes and proper metabolic regulation.

The central importance of these signaling and structural lipids is likely to explain the multitude of regulatory mechanisms modulating their levels. These include enzymes like ceramidases that break down ceramide (5, 6) and the LCBP lyase that cleaves these phosphorylated lipids into ethanolamine and a long chain aldehyde (7). More recent experiments in the yeast S. cerevisiae have identified a new membrane protein that is thought to efflux LCBS out of the cell. This protein has been designated Rsb1p (resistant to sphingoid bases) as it was identified as a high-copy suppressor of the PHS hypersensitivity of a dpl1Δ strain (8). Biochemical experiments provided evidence that Rsb1p stimulates the transport of radiolabeled dihydrosphingosine out of cells.

Further analyses of Rsb1p made an interesting connection between this putative transporter protein and multidrug resistance in S. cerevisiae (9). Disruption of the ATP-binding cassette transporter-encoding gene PDR5 elevated PHS tolerance and induced transcription of the RSB1 gene. PDR5 is a major determinant of multidrug resistance and is transcriptionally regulated itself by the zinc cluster-containing transcription factor Pdr5p (H9267) and Pdr3p (see Ref. 10 for a recent review).

Previous microarray experiments have indicated that PDR5 and RSB1 (systematic locus name YOR049c) are among the most highly induced transcripts by the presence of hyperactive alleles of either PDR1 or PDR3 (11, 12). Analysis of pdr5Δ cells argued that loss of this ABC transporter led to the induction of Pdr1p but not Pdr3p activity with accompanying elevation in RSB1 transcription (9). This transcription activation required the presence of at least one Pdr1p/Pdr3p response element (PDRE) located upstream of RSB1. Sucrose gradient experiments (9) coupled with previous indirect immunofluorescence experiments (8) led to the assignment of Rsb1p as an integral membrane protein of the endoplasmic reticulum.

We have previously found that two genes encoding enzymes involved in sphingolipid biosynthesis are transcriptionally responsive to loss of the mitochondrial genome (ρ0 cells). The signaling pathway linking nuclear gene expression with mitochondrial stress has been designated retrograde regulation (see Ref. 13 for a recent review). LAC1, encoding a component of ceramide synthase (14, 15), and IPT1, encoding the inositol phosphotransferase enzyme (16), are both transcriptionally up-regulated in ρ0 cells (17, 18). Importantly, ρ0 cells also strongly induce expression of both PDR5 and RSB1 in response to loss of the mitochondrial genome in a strictly Pdr3p-dependent fashion (19). Previous studies have established that ρ0 cells are highly resistant to drugs like cycloheximide because of this transcriptional induction of PDR5 (20–22). To determine whether retrograde signaling in ρ0 cells also leads to increased PHS tolerance, we have examined the PHS phenotype of ρ0 cells. Our experiments demonstrate that ρ0 cells induce RSB1 transcription in a Pdr3p-dependent fashion and exhibit a large increase in resistance to exogenous PHS challenge. Analyses of the RSB1 locus and protein indicate that the sequence deposited in the Saccharomyces Genome Database contains an error in the amino terminus that leads to the production of a truncated protein lacking 28 amino acids from the N terminus. A full-length form of Rsb1p containing these additional 28 residues localizes to the plasma membrane, and fully complements rsb1Δ cells, unlike the truncated membrane protein. Two potential
N-linked glycosylation sites are present in this 28-amin acid region and site-directed mutagenesis of these sites demonstrated that both must be present for normal Rsb1p function. These experiments provide new insight into the likely role of Rsb1p in control of PHS levels as well as illuminating the extensive interaction between the Pdr regulatory system and genes involved in sphingolipid homeostasis.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media—**The genotypes of the yeast strains used in this study are listed in Table 1. Yeast transformations were performed using the lithium acetate procedure (23). Cells were grown in cultures containingYPD (2% yeast extract, 1% peptone, 2% glucose) under non-selective conditions or appropriate SC media under selective conditions (24). PHS resistance was measured by spot test assay on plates with selective conditions or appropriate SC media under selective conditions resulting in the formation of colonies. The resulting PCR fragment was cloned into pCR2.1TOPO to form pSLP1. A 0.625-kbp fragment released on digestion with BamHI/XbaI was cloned in EcoRI-digested pSLP5, yielding pSLP2. All clones generated were sequenced to confirm their identities.

To introduce the *dpl1Δ* allele, we amplified the corresponding region from the genomic DNA of BY4742 *pdr1Δ::kanMX4* with primers cgggatccattaccttgatctcagctg-3 (rsb1-prom-del-1) having an EcoRI and a BamHI site, respectively. The EcoRI/BamHI-digested amplicon was then cloned into vector pSEYC102, which contains an *hisG* ORF beginning from this downstream ATG. The resulting PCR fragment was cloned into pCR2.1 TOPO to form pSLP1. A 0.625-kbp fragment released on digestion with BamHI/XbaI was cloned into pSLP5 digested with the same enzymes to construct the full-length ORF and produce pSLP3. This strategy results in both the uATG and Δ28 forms of Rsb1p being expressed from the natural ATG of *RSB1* but the Δ28 mutant lacks the wild-type Rsb1p residues corresponding to positions 2–28 in the primary translation product. To construct the wild-type *RSB1* clone in pRS316 without the BamHI site, primers cgggatccattaccttgatctcagctg-3 and 5′-cggctgtatgctctc-3′ with EcoRI and NotI sites were used to amplify the entire *RSB1* ORF and this PCR fragment was cloned into pCR2.1 TOPO to generate pSLP2. The 1.9-kbp fragment released on digestion of pSLP2 with EcoRI was cloned into EcoRI-digested pSLP5, yielding pSLP4. All clones generated were sequenced to confirm their proper construction.

The *RSB1* clones described above were all tagged at their COOH termini with a 3X hemagglutinin (HA) cassette using the method described by Longtine *et al.* (27). The forward primer contained 50 nucleotides of the COOH-terminal *RSB1* coding sequence followed by the F2 sequence (27), the reverse primer contained 50 nucleotides of pRS316 sequence followed by the R1 primer sequence. With these primers the 3X HA-*kanMX6* sequence was amplified from the corresponding pA6a plasmid and co-transformed into wild-type cells along with *RSB1* clones pSLP5 (Δ28 *RSB1*), pSLP3 (uATG *RSB1*), and pSLP4 (wt *RSB1*) linearized with NotI at the 5′-end of the *RSB1* ORF. Transformants were selected on medium lacking uracil and then tested for resistance (200 μg/ml). The 3X HA-tagged versions of the above clones were rescued from the above strain backgrounds and sequenced to confirm the fusion junction. The *RSB1* clones (pSLP3, pSLP4, and pSLP5) were also tagged with enhanced GFP (eGFP) using the method described previously by Gerami-Najad *et al.* (28).

**Construction of the RSB1 N-Glycosylation Mutants—**The asparagine residues at Asn-3 and Asn-6 were mutated to glutamine residues by site-directed mutagenesis using various primers. Primers incorporating

| Strain       | Genotype                  | Ref.     |
|--------------|---------------------------|----------|
| BY4742       | MATa his3-Δ1 leu2-Δ2 lys2-Δ3 ura3-Δ0 | Open Biosystems |
| YE6210 pΔ40  | MATa leu2-3,112 ura3-52 lys2-801 trp1-Δ901 his3-Δ200 suc2-Δ9 Mel− | Scott Ennr |

**Construction of the RSB1 N-Glycosylation Mutants—**The asparagine residues at Asn-3 and Asn-6 were mutated to glutamine residues by site-directed mutagenesis using various primers. Primers incorporating
Retrograde Regulation of Long Chain Base Resistance

the desired point mutations were synthesized and used for PCR amplification using Hi Fidelity Taq polymerase (Invitrogen). All the primers used had a BglII site placed immediately downstream of the ATG of the RSB1 ORF. The absence of the BglII site was used to confirm the generation of the final mutant clones. The reverse primer 5′-ATAAGAAATcgccgcccCAATAAGAGAGACCTGTGCG3′, with a NotI site used for amplification, was common for all the mutants. The following forward primers were used to generate the specific mutants: N3Q, 5′-ttccaaagtagtcgTCCCAAGCAAACAATATAC3′; N6Q, 5′-ttccaaagtagtcgTCCACACCGAACAACAAACTGTTGCG3′; and N3Q,N6Q, 5′-ttccaaagtagtcgTCCACAGCGACACAAATAATACGTTAGGCGAG3′. The wild-type RSB1 control for all the mutants also had a BglII site at the true ATG for RSB1 ORF and was amplified using the forward primer 5′-ttccaaATGagatctgTCCAACGCAACAAATATAC3′ and the common reverse primer to generate the clone pSLP29. PCR amplicons (384 bp) generated using the different forward primers in combination with the common reverse primer using pSLP35 as the template DNA were cloned into TOPO2.1vector. These clones were then digested with BglII and NotI and the 384-bp fragment was cloned into the BamHI/NotI-digested uATG RSB1 (pSLP35). They were all transferred to pSLP35 finally as a 1.0-kb KpnI fragment to generate the final clones, namely, pSLP28-N3,6Q-3 (pSLP35) and pSLP30, respectively.

Construction of the Reporter Plasmids—The wild-type RSB1-lacZ fusion plasmid (pTA2) was generated as described above. Overlap extension PCR mutagenesis was used to add the PDREs in RSB1 (29). Briefly, PCR were performed with a wild-type RSB1 template and two different mutagenic primers (mutant bases are in uppercase) for either PDRE 1 (gcaaatcttttaacTCCacagagacctgtccg) or PDRE 2 (gctcggactctttTCtagaGAaagatatggtctcc) and the primer rsb1-prom-del-1 (see above). These products were purified and mixed with a product generated using the primers −1000 RSB1for (see above) and PDREcom (CAACTATCGGAGCTGCCACA). Each pair of PCR products was then amplified using the two outside primers (−1000 RSB1for and rsb1-prom-del-1) with the resulting single mutant RSB1 promoter inserted into pSEYC102 as an EcoRI/BamHI fragment. A point mutation introduced in PDRE 2 generating a XbaI site yielded pTA5. Replacement of PDRE 1 with a XhoI restriction site yielded pGK3. A mutant plasmid lacking both PDREs (pGK2) was produced using pTA5 as a template and the PDRE 1 mutant primer (see above) along with the primer PDRE1XholantI (cgagacaaagatgctCTcagAagagatgtgcctcc) in a similar overlap extension strategy.

Immunoblotting—Cells were grown to an A600 of 1 and whole cell extracts were prepared by the TWIRL buffer (8 M urea, 5% SDS, 10% glycerol, and 50 mM Tris, pH 6.8) extraction method. 5 OD units of each sample were electrophoresed on SDS-PAGE, transferred to nitrocellulose, and Western blotted using monoclonal anti-HA (1:1000), polyclonal anti-Kar2p (1:10,000), and monoclonal anti-Pma1p (1:1000, Abcam) antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000) and anti-rabbit antibody (1:12,000) followed by measurement of chemiluminescence (Amersham Biosciences).

PNGase F Treatment—Crude cell extracts were prepared as described for sucrose density gradient fractionation from cells expressing various forms of HA-tagged Rsblp in ρ0 SEY6210. After centrifuging the cell extract at 2,000 × g for 3 min to remove unbroken cells, the supernatant was further centrifuged at 12,000 × g for 45 min. The membrane-enriched pellet was resuspended in 30 μl of Tris-HCl buffer, pH 7.5, + 5 μl of denaturation buffer + 5 μl of 10X protease inhibitor mixture (Roche) and incubated at 37 °C for 10 min. After denaturation, 5 μl of G7 buffer + 5 μl of Nonidet P-40 and 250 units of PNGase F were added. All the buffers including Nonidet P-40 were supplied by the manufacturer (New England Biolabs). Control samples were treated identically except that no PNGase F was added. The reaction mixtures were incubated at 37 °C for 16–24 h. After SDS-PAGE and transfer to a nitrocellulose membrane, the blot was probed with anti-HA antibody (1:1000). Primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000) followed by measurement of chemiluminescence (Amersham Biosciences).

RESULTS

PHS Resistance Depends on Mitochondrial Status—Previous DNA microarray experiments have suggested that RSB1 gene expression is strongly induced in S. cerevisiae cells lacking their mitochondrial genome (ρ0 status) (19, 21). To examine if ρ0 activation of RSB1 transcription can influence PHS tolerance, isogenic ρ+ and ρ0 strains were generated that varied according to the presence of the RSB1 gene. These strains were then compared for their relative PHS resistance by spot test assay (Fig. 1).

Wild-type, ρ+ cells were able to tolerate up to 10 μM PHS, whereas rsb1Δ strains failed to grow above 5 μM. Cells lacking the mitochondrial genome exhibited robust growth at 20 μM but this phenotype was dependent on the presence of the RSB1 gene. These data are consistent with the idea that transcriptional induction of RSB1 in ρ0 cells leads to PHS resistance. Other genes are likely to participate in this ρ0 elevation.

![FIGURE 1. Retrograde signals induce phytosphingosine resistance. Isogenic ρ+ and ρ0 cells containing or lacking RSB1 were grown to mid-log phase, and 1000 cells were placed at 3-μl spots on YPD media containing the indicated concentrations of PHS. Plates were incubated at 30 °C and then photographed to document growth of the cells.](image-url)
of PHS tolerance as the ρ^0 rsb1Δ cells continued to grow at 10 μM, a concentration ρ^+ rsb1Δ were unable to tolerate.

RSB1 Expression Is Induced in ρ^0 Cells in a Pdr3p-dependent Manner—The data described above are consistent with a model in which Pdr3p induces RSB1 transcription in ρ^0 cells leading to elevated PHS resistance. To test this model, we constructed a lacZ gene fusion to the putative ATG for the RSB1 open reading frame based on sequence information from SGD. This clone was introduced into isogenic ρ^+ and ρ^0 S. cerevisiae cells but we were unable to detect any β-galactosidase activity (data not shown). Comparison of the DNA sequences encoding the amino-terminal protein sequences from other Saccharomyces species suggested that the reported sequence for the S. cerevisiae RSB1 gene might contain an extra adenine residue 16 bp upstream from the predicted ATG. Removal of this adenine residue would extend the amino-terminal domain of Rsb1p by 28 residues that show strong sequence conservation with other fungal Rsb1p homologues. We directly sequenced this region of S. cerevisiae RSB1 and confirmed that the correct sequence lacked this adenine residue (data not shown). Additionally we constructed a lacZ gene fusion to the new ATG present at the start of the RSB1 ORF and found that this fusion expressed β-galactosidase activity. We have used this gene fusion as an indicator plasmid to evaluate RSB1 expression in the studies reported below.

This RSB1-lacZ reporter gene was introduced into a series of isogenic strains to evaluate RSB1 expression in response to loss of the mitochondrial genome and different alleles of PDR1 and PDR3. We also tested the PHS tolerance of these same strains (Fig. 2).

The RSB1-lacZ plasmid was induced in ρ^0 cells to 26 units/OD compared with 7 units/OD in ρ^+ cells. Removal of the PDR3 gene eliminated this ρ^-mediated induction. Similar to the behavior of other ρ^-induced genes regulated by Pdr3p (22), ρ^- pdr3Δ cells supported an even higher level of RSB1 expression than ρ^0 cells with expression rising to 35 units/OD. Loss of both PDR1 and PDR3 from ρ^0 cells caused no further decrease than that seen in ρ^- pdr3Δ strains alone.

These levels of RSB1 expression were generally consistent with the PHS tolerance of each strain. The ρ^- pdr1Δ cells were the most resistant to this LCB followed by ρ^+ cells. In ρ^- cells, the pdr1Δ pdr3Δ mutant was significantly more PHS sensitive than either single mutant strain. The pdr1Δ pdr3Δ mutant was also more sensitive to PHS (growth strongly inhibited at 5 μM) than the rsb1Δ strain. This suggests the existence of another gene regulated by the Pdr pathway that is involved in PHS tolerance. In support of this idea, the ρ^0 pdr1Δ pdr3Δ strain was more sensitive to PHS than the ρ^- pdr3Δ strain even though these two mutants drove essentially identical levels of RSB1-lacZ expression. This discrepancy is consistent with the possibility that a Pdr-regulated gene might be contributing to PHS tolerance along with RSB1.

We also examined the contribution of each of the two Pdr1p/Pdr3p response elements (PDREs) present in the RSB1 promoter. Site-directed mutations were constructed in the most upstream of the Pdr1p/Pdr3p response element (PDRE 1) or the more promoter-proximal binding site (PDRE 2) or both (PDRE 1 PDRE 2) in the context of the RSB1-lacZ fusion plasmid. These three mutant PDRE-containing plasmids were introduced into ρ^-, ρ^0, and pdr3Δ yor1Δ strains. Transformants were grown to mid-log phase and RSB1-dependent β-galactosidase activity was measured as described above. wt, wild-type.

The wild-type RSB1-lacZ fusion gene was induced by ~4-fold in ρ^- cells compared with a ρ^+ background. Loss of either PDRE strongly reduced ρ^- inducibility to less than 2-fold and the double mutant eliminated this weak residual ρ^- response. Both PDREs are required for the normal induction of RSB1 seen in ρ^0 cells.
Retrograde Regulation of Long Chain Base Resistance

We also transformed this series of RSB1-lacZ reporter genes into cells lacking the ABC transporters Pdr5p and Yor1p. Previous work of others (9) has suggested that loss of these transporters leads to the strictly Pdr5p-dependent activation of RSB1 expression. We did not observe any significant induction of RSB1-lacZ expression in this pdr5∆ yor1Δ strain (Fig. 3). Western blot analysis also failed to detect any increase in expression of a Rsb1p-3× HA construct (data not shown). Whereas this pdr5∆ yor1Δ strain did exhibit the elevated PHS resistance reported (9), there was no correlation with RSB1 expression in our experiments. The reason for this difference is unknown but may be a result of different strains and/or experimental protocols used for these experiments.

Characterization of Full-length and Mutant Rsb1p Forms—Because the data reported above indicate that the previously predicted form of Rsb1p is actually an amino-terminal truncation mutant, it was important to compare the properties of these two different Rsb1p derivatives.

To enable detection of Rsb1p, a 3× HA epitope tag was added to its extreme carboxyl terminus. The wild-type RSB1 gene was prepared by PCR amplification using primers that bound ~1000 bp upstream of the ATG and 500 bp downstream of the stop codon. This fragment was cloned into a low-copy number plasmid and the 3× HA tag was inserted in place of the natural termination codon.

Along with this wild-type form of RSB1, we also produced several mutant versions of this gene. A truncation mutation lacking the amino-terminal 28 residues, corresponding to the previously characterized form of Rsb1p (8), was prepared and designated Δ28 RSB1. Inspection of the sequence of these 28 amino acids also indicated the presence of two potential N-linked glycosylation sites located at residues 3 and 6 in full-length Rsb1p. Single substitution mutations were generated that removed each site individually (N3Q and N6Q Rsb1p) as well as a double mutant that lacked both sites (N3Q,N6Q Rsb1p). As for the wild-type gene described above, all these mutants were tagged with 3× HA and carried on a low-copy number plasmid under control of the wild-type RSB1 promoter. These Rsb1p derivatives were introduced into yeast cells and analyzed for their expression and function. We also constructed carboxyl-terminal eGFP fusions to the wild-type, N3Q, N6Q, and Δ28 forms of Rsb1p to compare the localization of these proteins.

Expression of Rsb1p Forms—Western blotting experiments were carried out to determine the expression profile of each Rsb1p form in ρ+ and ρ− cells. An empty vector plasmid was introduced as a specificity control for anti-HA blotting. Whole cell protein extracts were prepared and equal aliquots were electrophoresed through SDS-PAGE, followed by Western blot analysis (Fig. 4).

Two different forms of wild-type Rsb1p were detected by anti-HA immunoblotting: a single species of ~43 kDa and a larger, less discrete form of apparent molecular mass ranging from 64 to 80 kDa. Interestingly, the Δ28 Rsb1p form exhibited a single HA immunoreactive species of 40 kDa. The double substitution mutant form of Rsb1p lacking both putative N-glycosylation sites (N3Q,N6Q RSB1) produced a doublet around 43 kDa but no proteins above this molecular mass. The mutant lacking the most amino-terminal of the putative glycosylation sites (N3Q RSB1) generated a disperse group of immunoreactive proteins around 60 kDa and a single polypeptide of 43 kDa. Interestingly, the other single glycosylation site mutant (N6Q RSB1) produced lower levels of the 60-kDa group of proteins but an increased amount of the doublet at 43 kDa. All these proteins were more abundant in samples prepared from ρ− cells indicating that retrograde regulation was still operational on all the mutant genes. Vph1p immunoblotting indicated that comparable levels of protein were present in each sample.

This Western blot analysis provided several important new pieces of information relevant to the biogenesis of Rsb1p. First, full-length Rsb1p produces two distinctly different immunoreactive species: a heterodisperse form ranging from 64 to 80 kDa and a more discrete form at 43 kDa. Second, expression of a mutant form of Rsb1p lacking the amino-terminal 28 residues produces a single species of 40 kDa. Third, inactivation of two putative N-linked glycosylation sites present in this amino-terminal 28-residue region prevents the formation of the heterodisperse forms of Rsb1p. Finally, loss of either potential N-linked glycosylation site individually led to production of a new high molecular mass group of immunoreactive species grouped around 60 kDa. Given that these mutant forms of Rsb1p produced a range of polypeptides when present in cells, we assessed the function of each by testing for their ability to complement the PHS sensitivity of a rsb1Δ dpl1Δ strain.

Reduced Function of Mutant Forms of Rsb1p—Low-copy number, URA3-containing plasmids expressing wild-type and mutant forms of Rsb1p derivatives were transformed into a rsb1Δ dpl1Δ strain. We used this strain as a sensitized genetic background because PHS toxicity depends on interference with nutrient transporter function (31, 32). Work from other labs has shown that complementation of auxotrophic markers in S. cerevisiae cells reduces the sensitivity of these strains to PHS (31) and inclusion of the dpl1Δ lesion facilitates detection of the PHS-sensitive phenotype (8). Transformants were placed on minimal medium containing different concentrations of PHS and their ability to tolerate this long chain base was evaluated (Fig. 5).

Introduction of a plasmid expressing the wild-type form of Rsb1p restored the ability of transformants to grow in the presence of 5 and 7.5 μM PHS. The Δ28 Rsb1p derivative exhibited the greatest defect in PHS resistance although it still maintained some ability to function. Loss of either N-glycosylation site individually reduced the ability of the resulting protein to function compared with wild-type. The double replacement mutant (N3Q,N6Q Rsb1p) was slightly more defective than either single mutant but still superior to the Δ28 form of Rsb1p. Together with the Western blot analysis, these data support the view that Rsb1p must be glycosylated to exhibit full function.

Localization of Wild-type and Mutant Rsb1p—Previous studies using the Δ28 form of Rsb1p have suggested that this protein is found in the endoplasmic reticulum (8, 9). eGFP fusions to wild-type, Δ28, and N3Q,N6Q Rsb1p were prepared to evaluate the subcellular distribution of these proteins. The addition of eGFP to the COOH termini of these Rsb1p derivatives did not alter their relative ability to complement the
PHS hypersensitivity of a rsb1Δ dpl1Δ strain (data not shown). Low-copy number plasmids expressing the Rsb1p-eGFP fusions were introduced into ρ− and ρ0 cells. Transformants were grown to mid-log phase and visualized by fluorescence microscopy (Fig. 6). Cells were also stained with FM4-64 to ascertain the location of the vacuole as described (33).

All Rsb1p forms were found at the periphery of the cell, consistent with a plasma membrane localization in both ρ− and ρ0 cells. The fluorescence intensity was higher in the ρ0 background but no significant difference in localization was seen. Importantly, all three forms of Rsb1p were also found to accumulate intracellularly. This can be most clearly seen in ρ0 cells where the intracellular eGFP signal overlaps with the FM4-64 fluorescent signature of the vacuolar membrane. Many S. cerevisiae proteins that are localized on the plasma membrane are often routed to the vacuole for their degradation (recently reviewed in Ref. 34). These data suggest this is likely to be true for Rsb1p but further experiments are required to confirm this idea. The central conclusion is that the mutant forms of Rsb1p analyzed here exhibit plasma membrane location that is indistinguishable from the wild-type protein.

To confirm the results using the Rsb1p-eGFP fusions, sucrose gradient fractionation was employed to examine the distribution of epitope-tagged Rsb1p proteins between different membrane compartments. Isogenic ρ− and ρ0 strains expressing either wild-type or Δ28 Rsb1p-3×HA proteins were grown to mid-log phase and whole cell lysates were prepared. Lysates were separated by sucrose density centrifugation and fractions were collected from these gradients. Aliquots of each fraction were electrophoresed on SDS-PAGE and analyzed by Western blotting with anti-HA and marker antibodies (Fig. 7).

In ρ− cells, wild-type Rsb1p was found to enrich in the densest (bottom) of the gradient in a manner overlapping with Pma1p, the plasma membrane ATPase (35). Both the large and smaller forms of wild-type Rsb1p appeared to fractionate in a similar fashion. Importantly, both of these forms of Rsb1p fractionated with behavior distinct to that of the ER chaperone, Kar2p, which was enriched in the center of these gradients. Strikingly, the Δ28 form of Rsb1p was also found to enrich in the bottom of the gradient along with Pma1p. This finding suggests that the reduced function seen for this mutant form is unlikely to be explained by a defect in localization. Both wild-type and Δ28 forms of Rsb1p were expressed at higher levels in the fractions from ρ0 cells but no changes in their subcellular distributions were detected.

These localization data provide independent support for the assignment of Rsb1p as a component of the S. cerevisiae plasma membrane.

Additionally, the functional defect of the Δ28 form of Rsb1p is unlikely to be a consequence of mislocalization.

Wild-type but Not Δ28 Rsb1p Is Glycosylated—The appearance of the higher molecular mass form of Rsb1p in cells expressing the wild-type but not Δ28 or N3Q,N6Q forms of this protein suggested the possibility that the longer Rsb1p derivative was glycosylated. To test this idea, membranes were prepared from cells expressing the wild-type, N3Q,N6Q, or Δ28 Rsb1p derivatives and subjected to PNGase F digestion. PNGase F will cleave N-linked sugars from their protein backbones (36). After PNGase F digestion, aliquots were resolved on SDS-PAGE and analyzed by Western blotting with anti-HA antibodies (Fig. 8).

As seen above, wild-type Rsb1p produced two different forms of immunoreactive protein: a heterogeneous, higher molecular mass species and a more discrete smaller form. PNGase F digestion resulted in an elimination of the heterogeneous species with an increase in the intensity of a band with slightly higher molecular weight as compared with the discrete, lower molecular weight form that is thought to represent deglycosylated Rsb1p. The difference in molecular mass between these two smaller Rsb1p digestion products may represent partial deglycosylation but is not currently understood. Conversely, no such change in electrophoretic behavior was seen for either the N3Q,N6Q or the Δ28 Rsb1p derivatives. These data support the conclusion that most, but not all, wild-type Rsb1p is a glycoprotein, whereas no glycosylation could be found in the case of the Δ28 or the N3Q,N6Q mutants. Two consensus sites (Asn-X-Thr/Ser) for N-linked glycosylation (37) can be found that begin at residues 3 (NAT) and 6 (NNT). As described above, expression...
Retrograde Regulation of Long Chain Base Resistance

FIGURE 7. Sucrose gradient analysis of membrane distribution of Rsb1p. Isogenic ρ⁺ and ρ⁰ cells were transformed with low-copy number plasmids carrying RSB1–3×HA fusion genes corresponding to the wild-type and Δ28 forms of this protein. Transformants were grown to mid-log phase and whole cell lysates were prepared. These lysates were centrifuged through a 10–60% sucrose cushion at 300,000 × g for 12 h. Equal aliquots of gradient fraction were then collected and concentrated with the addition of trichloroacetic acid. The resulting precipitates were resuspended in loading buffer and separated by SDS-PAGE. After transfer to nitrocellulose membranes, the distribution of proteins was analyzed by Western blotting with the indicated antibodies. Top refers to the least dense fraction of the gradient, whereas bottom indicates the fraction from the densest part of the gradient. The position of glycosylated Rsb1p is indicated by the arrow. Antibodies to Kar2p (endoplasmic reticulum) and anti-Pma1p (plasma membrane) antibodies were used as controls for the distribution of proteins of known subcellular location.

FIGURE 8. The full-length but not Δ28 form of Rsb1p is glycosylated. Microsomal membranes were prepared from ρ⁰ cells expressing wild-type, N3Q, N6Q, or Δ28 Rsb1p under control of the RSB1 promoter. All Rsb1p forms were expressed from a low-copy number plasmid and contained a 3×HA epitope tag at their COOH terminus. These membranes were then digested at 37 °C in the presence (+) or absence (−) of PNGase F for 16–24 h. Aliquots were electrophoresed through SDS-PAGE and analyzed by Western blotting with a monoclonal antibody directed against the HA epitope. Molecular mass standards are indicated on the left side in kDa.

of either a N3Q or N6Q mutant form of Rsb1p produced two immuno-reactive forms of Rsb1p: a series of proteins centered around 60 kDa and a polypeptide of 43 kDa. We subjected samples from cells expressing these single mutant derivatives to PNGase F digestion and found that the 60-kDa series of proteins were sensitive to digestion by this glycosidase. The simplest interpretation of these data is that Rsb1p is glycosylated at both the Asn-3 and Asn-6 positions with this dually glycosylated form appearing around 80 kDa.

DISCUSSION

Loss of the mitochondrial genome triggers global reprogramming of gene expression called the retrograde response (13). Several laboratories have provided evidence that ρ⁰ cells dramatically induce expression of ABC transporter-encoding genes like PDR5 along with elevation of transcription of loci encoding enzymes involved in sphingolipid biosynthesis (19–21, 38). This work extends the link between retrograde regulation and sphingolipid biosynthesis with the identification of RSB1 as a target of Pdr3p regulation in ρ⁰ cells. Establishing that ρ⁰ cells induce RSB1 via a Pdr3p-dependent mechanism provides further support to the idea that the physiological role for the Pdr regulatory pathway in S. cerevisiae is to modulate the lipid composition of membranes.

Along with this new insight into the transcriptional control of RSB1, several features of the localization of Rsb1p have been clarified. First, we have found and corrected a sequencing error that led to the prediction of an amino-terminal truncated Rsb1p (8). Correction of this error extends the region of sequence similarity with other Saccharomyces species and more importantly is required for the normal glycosylation of Rsb1p to occur. Second, complementation assays indicate that these additional 28 residues are required for normal function of Rsb1p. Third, two distinct N-linked glycosylation sites are found in this amino-terminal region of Rsb1p. Finally, analyses of subcellular localization indicate that full-length, N3Q, N6Q, or the Δ28 forms of Rsb1p accumulate in the plasma membrane, a location that simplifies the view of Rsb1p action given that this protein has previously been demonstrated to lead to efflux of LCBS out of the cell (8).

Several of the conclusions reported here differ from the previous work of Kihara and Igarashi (8, 9). These authors assigned Rsb1p an endoplasmic reticulum localization and did not report any glycosylation of this protein. There are two likely reasons to explain the different conclusions reached by these authors. First, their Rsb1p expression constructs were all based on the incorrect DNA sequence and led to the production of the truncated form of Rsb1p lacking the N-linked glycosylation sites. Second, amino-terminal epitope-tagged forms of Rsb1p were employed that could interfere with the function of Rsb1p. Our expression constructs employed full-length Rsb1p with carboxyl-terminal tags that were demonstrated to normally complement rsb1Δ strains.

S. L. Panwar and W. S. Moye-Rowley, unpublished data.
biosynthesis (17, 18). This study and the work of Kihara and Igarashi (9) place the RSRI gene clearly in the Pdr regulon. In mammalian cells, ABC transporters have been linked to transbilayer distribution of phospholipids, cholesterol, and sphingolipids (52). Because signals that activate the Pdr pathway (such as in \( \rho_0 \) cells) simultaneously induce ABC transporters like Pdr5p and a variety of sphingolipid homeostatic proteins, it is tempting to speculate that Pdr1p- and Pdr3p-mediated transcriptional regulation acts to coordinate biosynthesis and distribution of a variety of membrane components. Future work will be directed toward testing this hypothesis.

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Retrograde Regulation of Long Chain Base Resistance

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