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Suppressor Gene Analysis Reveals an Essential Role for Sphingolipids in Transport of Glycosylphosphatidylinositol-Anchored Proteins in *Saccharomyces cerevisiae*

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Sphingolipids are normally necessary for growth of *Saccharomyces cerevisiae* cells, but mutant strains that bypass the need for sphingolipids have been identified. Such bypass mutants fail to grow under stressful conditions, including low pH (pH 4.1), when they lack sphingolipids. To begin to understand why sphingolipids seem to be necessary for coping with low-pH stress, we screened a genomic library and selected a suppressor gene, *CWP2* (cell wall protein 2), that when present in multiple copies partially compensates for the lack of sphingolipids and enhances survival at low pH. To explain these results, we present evidence that sphingolipids are required for a normal rate of transport of glycosylphosphatidylinositol (GPI)-anchored proteins, including Cwp2 and Gas1/Gpg1, from the endoplasmic reticulum (ER) to the Golgi apparatus. The effect of sphingolipids is specific for transport of GPI-anchored proteins because no effect on the rate of transport of carboxypeptidase Y, a non-GPI-anchored protein, was observed. Since the Gas1 protein accumulated in the ER with a GPI anchor in cells lacking sphingolipids, we conclude that sphingolipids are not necessary for anchor attachment. Therefore, sphingolipids must be necessary for a step in formation of COPII vesicles or for their transport to the Golgi apparatus. Our data identify the Cwp2 protein as a vital component in protecting cells from the stress of low pH.

Sphingolipids comprise a complex class of molecules found primarily in the plasma membrane of eucaryotic cells. Sphingolipids have been shown to play roles in many cellular processes, including cell-to-cell recognition, differentiation, and growth regulation (reviewed in references 1, 20, 21, 26, and 33). Possible new roles for sphingolipids have appeared in growing numbers in the recent published literature, with much attention being given to the role of sphingolipid breakdown products as second messengers in signal transduction pathways mediating, for example, apoptosis (7, 17, 19, 22) and growth control (5, 13, 24, 32, 37, 43). Our research has focused on uncovering new functions for sphingolipids by using mutant strains of *Saccharomyces cerevisiae* that can be manipulated so as to contain or lack sphingolipids. The utility of this approach is demonstrated herein by experiments showing that sphingolipids are necessary for transport of proteins with a glycosylphosphatidylinositol (GPI) lipid anchor from the endoplasmic reticulum (ER) to the Golgi apparatus.

Cell surface proteins anchored to the plasma membrane by linkage to GPI are found in many eucaryotic cells. Such anchored proteins include receptors, adhesion molecules, lymphoid antigens and antigens of unknown function, hydrolytic enzymes, the scrapie prion protein, and the variant surface glycoproteins of some parasitic protozoans (16, 47). GPI-anchored proteins are essential for the viability of *S. cerevisiae* cells (28) and are known to comprise a major component of the cell wall (31). Synthesis of the GPI moiety and attachment to the carboxy terminus of a protein occur in the ER followed by vesicular transport to the Golgi apparatus and then to the plasma membrane (47).

Wild-type *S. cerevisiae* strains require sphingolipids for viability (51), but viable mutant strains lacking sphingolipids have been isolated (14). Such strains, e.g., 4R3, contain two mutated genes. One is a deletion of the *LCB1* or *LCB2* gene, both of which encode subunits of serine palmitoyltransferase (34), the initial enzyme in sphingolipid synthesis. Mutation of either *LCB* gene creates a nutrient requirement for a sphingoid long-chain base (Lcb phenotype) such as phytosphingosine (PHS). The second mutation produces a suppressor gene, *SLC1-1*, that bypasses the requirement for exogenous PHS and for sphingolipids (35). The suppressor gene enables cells to produce a novel set of glycerophospholipids that mimic sphingolipid structures and probably some of their functions (30). The novel lipids are phosphatidylinositol (PI), mannosyl-PI, and inositol-P-(mannosyl-PI), all containing a C_{26} fatty acid in the *sn*2 position of the glycerol moiety. Normally the C_{26} fatty acid is found only N-acylated to PHS to give phytoceramide, which is modified in a stepwise manner to yield the normal sphingolipids inositol-P-ceramide (IPC), mannose-inositol-P-ceramide, and mannose(inositol-P)$_2$-ceramide.

Strains such as 4R3 have impaired stress responses when they lack sphingolipids and are unable to grow at pH 4, at 37°C, or in the presence of 0.75 M sodium chloride, whereas normal yeast cells and 4R3 cells containing sphingolipids readily grow under these conditions (38). To begin to understand why sphingolipids are necessary for growth at low pH, a yeast recombinant DNA library was screened for a suppressor gene that would enable cells to survive and to grow at pH 4 in the absence of sphingolipids, the rationale being that an increase in the concentration of a specific protein might compensate for the lack of sphingolipids. This approach yielded the *CWP2* (cell wall protein 2) gene, which encodes a major mannoprotein component of the outer layer of the yeast cell wall (50). Our

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TABLE 1. Genotypes and origins of yeast strains used in this study

Strain	Genotype	Relevant phenotype or gene	Reference or source
SJ21R	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1*	Parent of 4R3, Lcb^+	25
1Δ4	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1 lcb1::URA3*	*lcb1* deleted from SJ21R, Lcb^-	25
4R3	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1 lcb1::URA3 SLC1-1*	Derived from 1Δ4, Lcb^+ *SLC1-1* suppressor	14
MS100	*MAT***a** *leu2-3,112 ade1, MEL1 cwp2-Δ1 lcb1::URA3 SLC1-1*	Derived from 4R3, *cwp2* deleted	This work
MS106	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1 CWP2::LEU2 lcb1::URA3 SLC1-1*	4R3 with 6 copies of *CWP2*	This work
MS200	*MAT***a** *ura3-52 leu2-3,112 ade1 MEL1 cwp2-Δ1*	SJ21R with *cwp2* deleted	This work
W303-1B	*MATα ade2-1 can1-100 his 3-11,15 leu2-3,112 trp1-1 ura3-1*		R. Rothstein
WB2d	*MATα ade2-1 can1-100 his 3-11,15 leu2-3,112 trp1-1 ura3-1 gas1::LEUs*	W303-1B with *gas1* deleted	40

data support the hypothesis that sphingolipids, perhaps ceramide itself, are essential for a normal rate of transport of GPI-anchored proteins, including Cwp2, from the ER to the Golgi apparatus. In the absence of sphingolipids, transport of Cwp2 to the cell wall is impaired, resulting in a cell wall that fails to protect cells from the stress of low pH.

MATERIALS AND METHODS

Strains, growth conditions, and plasmids. The yeast strains used in these experiments are listed in Table 1. Strains with the *cwp2-Δ1* allele were made by using a published single-step gene transplacement procedure (42). The *cwp2-Δ1* allele has the region between the *Sac*II site (−469 relative to the ATG start codon) and the *Nsi*I site (316 bp downstream from the end of *CWP2*) replaced with a 1.5-kb *Sma*I-*Pst*I DNA fragment carrying the *LEU2* gene. Strain MS106 was made by transforming 4R3 cells with pMS30XH-1 DNA, linearized by digestion with *Sac*II to promote integration at the *Sac*II site located 469 bp upstream of *CWP2*. Leu^+ transformants were selected on plates containing PHS. Analysis of a Southern blot made by using genomic DNA digested with *Xba*I identified a transformant, MS106, whose DNA hybridized five to six times more strongly to the radioactive probe (determined by densitometry) than the parental strain, indicating that MS106 contains five to six tandem copies of the transforming plasmid. In addition, Southern blot analysis of MS106 chromosomal DNA digested with a restriction endonuclease, *Sac*I, that did not cleave within pMS30XH-1 (7.5 kb) showed a single hybridizing band of greater than 30 kb, indicating that the copies of pMS30XH-1 are in tandem. pMS30XH-1 has a 2.5-kb *Hin*dIII-*Xba*I genomic DNA fragment containing *CWP2* cloned into a derivative of pRS305 (44) having its *Sac*II site removed.

Construction of chimeric genes was facilitated by introduction of an *Eco*RI restriction site into *GAS1* and *CWP2*. The GAS^{EcoRI} allele has an *Eco*RI site at position 1992 which changed Ala-507 to Ser. It was made, using a synthetic oligonucleotide and the PCR (2), by changing G-1996 to T. GAS^{stop} has a stop codon at position 507 and was made by partially cutting a plasmid carrying GAS^{EcoRI}, filling the ends by using Klenow DNA polymerase, and ligating. The chimeric *GAS-CWP* gene was constructed by replacing the 107-bp *Eco*RI-*Hin*dIII fragment encoding the C terminus of the GAS^{EcoRI} gene with a 388-bp *Eco*RI-*Hin*dIII fragment encoding the C terminus of the CWP^{EcoRI} gene. The CWP^{EcoRI} gene encodes Ser instead of Gly-72 because of four base changes (A-209 to G, C-212 to T, G-213 to T, and G-214 to C) which were made by using PCR and a mutagenic oligonucleotide (5′-ATCTCTCAACAAACTGAGAATT CCGCTGCTAAGGCCGCTGTCG-3′). CWP^{stop} has a stop codon at position 72 and was made by filling in of the *Eco*RI ends (by using Klenow DNA polymerase) followed by ligation. The *CWP-GAS* gene was constructed by replacing the 388-bp *Eco*RI-*Hin*dIII fragment of CWP^{EcoRI} with a 107-bp *Eco*RI-*Hin*dIII fragment obtained from GAS^{EcoRI}. PCR products were verified by DNA sequence analysis. *GAS1* and *CWP2* alleles were cloned into the *CEN* vector pRS316 (44).

Yeast strains were grown on modified PYED (PYED buffered to pH 5.0 [PYED-5.0]), which contained 1% yeast extract (Difco), 1% Bacto Peptone (Difco), 2 or 4% glucose, 50 mM sodium succinate (pH 5.0), inositol (50 mg/liter), and potassium phosphate monobasic (500 mg/liter) or on defined medium supplemented as described previously (9) and containing, when necessary, 25 μM PHS in PYED and 12.5 μM in defined medium. PYED buffered to pH 4.1 (PYED-4.1) was made by mixing 290 ml of autoclaved agar (2% for plates only), 300 ml of autoclaved yeast extract plus peptone, 100 ml of filter-sterilized glycylglycine (0.5 M, pH 3.1), 200 ml of glucose (20%), 10 ml of inositol (0.5%), and 100 ml of potassium phosphate monobasic (0.5%).

Miscellaneous procedures and materials. Sensitivity to glucanase was tested as described previously (50) except that yeast lytic enzyme (70,000 U/g; ICN Biochemicals, Irvine, Calif.) was used in place of Zymolyase.

Anti-yeast carboxypeptidase Y (CPY) monoclonal antibody (catalog no. 10A-B5) was from Molecular Probes (Eugene, Ore.), and rabbit polyclonal anti-Gas1 antibodies were a gift from L. Popolo and L. Alberghina (48). For Western blot analysis, proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (8% gel) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, Mass.), using a semidry electrophoretic transfer apparatus (Bio-Rad, Hercules, Calif.). Blots were blocked in BLOTTO (5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20). Antibodies were used at a dilution of 1:3,000 (Gas1) or 1:1,000 (CPY) in BLOTTO. Bound antibodies were detected by enhanced chemiluminescence (Amersham International, Amersham, England) and exposure to X-ray film. The relative intensities of bands on X-ray film were quantified by using SigmaGel software (Jandel Scientific Software, San Raphael, Calif.).

Lcb^- strains were starved for PHS by using a modified version of a published procedure (39). Cells were grown at 30°C in PYED containing 12.5 μM PHS and 0.05% Tergitol to an optical density at 600 nm (OD_{600}) of about 0.5, harvested by centrifugation at room temperature, rinsed three times with an equal volume of PYE containing 0.05% Tergitol and 20 mM α-cyclodextrin (U.S. Biochemical, Cleveland, Ohio), and resuspended in PYED containing 0.05% Tergitol. Cells were aerated at 30°C, treated with 10 mM NaN_3 and 10 mM NaF at the indicated time points, chilled on ice, and processed as described for the pulse-chase labeling and immunoprecipitation procedure to give a cell extract.

The presence of a GPI anchor on the Gas1 protein was detected by using a published procedure (15), with slight modifications. Protein extracts were diluted with TEP (100 mM Tris-HCl [pH 7.5], 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) to a final volume of 500 μl and 4 mg of protein/ml. Samples were mixed with 55 μl of 10% (wt/vol) Triton X-114 in TEP, incubated on ice for 30 min, and centrifuged in a microcentrifuge for 5 min at 4°C. The supernatant fluid was warmed for 5 min at 32°C and centrifuged for 20 s at room temperature to separate the phases. The aqueous and detergent phases were reextracted three times with 1% Triton X-114 and TEP, respectively, and the volume of both phases was adjusted to 500 μl with TEP. Proteins were precipitated by treatment with trichloroacetic acid (10% [vol/vol]) on ice for 1 h, centrifuged for 10 min at 4°C, washed three times with 500 μl of acetone at room temperature, air dried, and resuspended in 2× Laemmli loading buffer (27) by incubation at 75°C for 15 min.

For treatment with PI-specific phospholipase C (PI-PLC; a gift from S. Turco [10]), protein extracts were diluted to a volume of 200 μl and a concentration of 10 mg/ml with TEP containing protease inhibitors (2 μl each of pepstatin [1.7 mg/ml], leupeptin [2.5 mg/ml], and aprotinin [1.7 mg/ml]; all from Sigma Chemical Co., St. Louis, Mo.). Samples were incubated overnight at 37°C with 2 μl of PI-PLC, diluted to 500 μl with TEP, and extracted with Triton X-114.

Pulse-chase labeling and immunoprecipitation. Cells were grown overnight to an OD_{600} of 0.7 in defined medium containing or lacking 5 μM PHS, harvested by centrifugation, rinsed once with medium lacking methionine, and resuspended in the same medium to a density of about 5×10^7 cells per ml. After 5 min of incubation at 30°C, ^{35}S Translabel (ICN) was added to final concentration of 100 μCi/ml, and labeling continued for 5 min. Radiolabel was diluted by adding methionine and cysteine to 30 mg/ml and ammonium sulfate to 3 mM. At the indicated time points, 1-ml samples were taken, supplemented with NaF and NaN_3 to 10 mM (final concentration), and chilled on ice. Cells were rinsed with medium, resuspended in 200 μl radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Tergitol, 0.5% deoxycholate, 1% SDS, 1% 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride), and broken by vortexing (five times, 30 s each) with 0.5-mm-diameter glass beads at 4°C. Samples were boiled for 5 min and cleared by centrifugation for 10 min at $10{,}000 \times g$. A portion (40 μl) of the extract was diluted 25-fold in RIPA buffer without SDS and 2-mercaptoethanol, incubated on ice for 30 min, and centrifuged at $10{,}000 \times g$ for 10 min to remove any nonspecific precipitate. The supernatant fluid was mixed with 10 μl of anti-Gas1 or 5 μl of anti-CPY antibody and incubated at 4°C overnight. Immune complexes were absorbed on protein A-agarose beads (50 μl of 10% [wt/vol]; Sigma) for 2 h at 4°C with constant mixing and recovered by centrifugation. After five washes in RIPA buffer without SDS and 2-mercaptoethanol, the complexes were suspended in 20 μl of SDS-PAGE loading buffer and boiled for 5 min. A 10-μl portion was subjected to SDS-PAGE (8% gel), and a fluorograph of the gel was prepared (6).

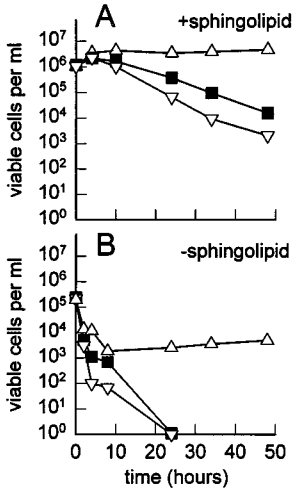

FIG. 1. *CWP2* and sphingolipids enhance cell survival at low pH. Cells were grown at 30°C overnight to an OD_{600} of 0.5 in PYED-5.0 containing (A) or lacking (B) 25 μM PHS, diluted into PYED-4.1 to about 10^6 cells/ml, and incubated. At the times shown, samples were sonicated to disaggregate clumped cells, diluted, spread on PYED-5.0 agar plates containing 25 μM PHS, and incubated at 30°C to determine viable cells. Symbols: ■, 4R3 (*CWP2*); ▽, MS100 (*cwp2-Δ1*); △, MS106 (six copies of *CWP2*).

RESULTS

Isolation of a gene that enhances survival at pH 4 in the absence of sphingolipids. To begin to understand why sphingolipids are necessary for growth of cells at low pH, we screened a genomic DNA library carried in pRS315 (*CEN4 LEU2* [44]) for a gene that would promote growth, on agar medium having a pH of 4.1 (PYED-4.1), of transformed 4R3 cells lacking sphingolipids. After 4 days incubation at 30°C, colonies resistant to low pH (Lpr^r) were obtained. Plasmid DNA isolated from several Lpr^r colonies carried the same gene, *CWP2*, which when retransformed into 4R3 cells produced over a 1,000-fold increase in the frequency of Lpr^r cells (unpublished data).

To examine this phenomenon further, isogenic derivatives of strain 4R3 containing no and six tandem, chromosomally integrated copies of the *CWP2* gene were constructed, and the frequency at which the Lpr^r phenotype arose was measured. Strain MS100 (no *CWP2* gene) produced Lpr^r colonies at a frequency of 4.6×10^{-8}. Strain 4R3 (one *CWP2* gene) yielded Lpr^r colonies at a higher frequency (5.9×10^{-6}), and strain MS106 (six copies of the *CWP2* gene) showed an even higher frequency (2.8×10^{-4}). These data suggest that cells with more copies of the *CWP2* gene survive longer at low pH (see below). Increased survival enhances the opportunity for emergence of a mutation (unpublished data) which enables cells to grow.

The hypothesis that *CWP2* enhances survival was examined by measuring the viability of strains carrying no, one, or six *CWP2* genes during incubation at pH 4.1. As predicted, viability was enhanced by increasing the dosage of *CWP2* in cells lacking sphingolipids. For example, the strain with six copies of the gene gave a 100-fold loss of viability within 10 h but thereafter showed no further loss (Fig. 1B). After 3 to 4 days, growth resumed (data not shown). In contrast, the strains with no or one *CWP2* gene gave more than a 10^5-fold loss of viability by 20 h of treatment, and growth did not resume. When the same cells contained sphingolipids, they did not lose viability for at least 5 h of low-pH treatment (Fig. 1A). After 5 h, the strain with six copies of *CWP2* did not lose viability at all, while the other two strains lost viability slowly but not nearly to the same extent as they did when sphingolipids were absent. We conclude that viability is enhanced by the *CWP2* gene at pH 4.1; this effect is most pronounced in cells lacking sphingolipids.

Cells lacking sphingolipids have a reduced Cwp2 protein content. One explanation for a reduction in viability at low pH in cells lacking sphingolipids and an enhancement of viability by increased dosage of the *CWP2* gene is that one or more steps in transport of GPI-anchored proteins to the plasma membrane or cell wall require sphingolipids. Without sphingolipids, the rate of transport of the putative GPI-anchored Cwp2 protein to the cell wall is reduced, yielding a less protective cell wall and reducing viability. Increased dosage of the *CWP2* gene increases its rate of transport, perhaps by mass action, so that the Cwp2 protein content of the wall increases and protects cells from protons. To test this hypothesis, we attempted to directly measure the Cwp2 protein content of cells by using a published procedure (50) but were unable to detect the protein in a glucanase (laminarinase)-extracted cell wall fraction. A possible reason for a lack of detection is heterogeneity in glucanase preparations which are an ill-defined mixture of enzymes.

As an alternative to direct measurement of the Cwp2 protein content of cells, we used an indirect assay which measures the susceptibility of a cell suspension to lysis by treatment with glucanase. The rationale for this assay is that the thicker the outer mannoprotein layer of the cell wall, where the Cwp2 protein is located, the greater the protection of the inner glucan layer from glucanase digestion. Once digestion of the glucan layer reaches a critical point, cells lyse and the absorbance of the sample decreases. Using this assay, van der Vaart et al. (50) demonstrated that wild-type cells are more resistant to digestion than an isogenic strain lacking the *CWP2* gene. Using our wild-type parental strain (SJ21R) and a derivative lacking *CWP2* (MS200), we obtained the same kinetics of cell lysis as did van der Vaart et al. (Fig. 2A). Thus, resistance to glucanase digestion appears to be a reliable way to compare the Cwp2 protein contents of strains.

Using the glucanase digestion assay, we measured the apparent Cwp2 protein content of cells lacking sphingolipids. Cells lacking the *CWP2* gene were the least resistant to digestion, cells with one copy of the gene were slightly more resistant, and cells with six copies of the gene were the most resistant (Fig. 2B). All three strains were more resistant to digestion when they contained sphingolipids, and under this growth condition, the strain with six copies of *CWP2* was always more resistant than any of the other strains (Fig. 2C), including the wild-type parent (data not shown). We also measured the *CWP2* mRNA level by Northern blotting. The mRNA was absent in the deletion strain (MS100) and five to six times more abundant in the strain with six copies of the *CWP2* gene than in the strain with one copy (data not shown). Taken together, these data imply that the Cwp2 protein content of the outer cell wall layer is reduced in cells lacking sphingolipids. This reduction can be partially reversed by increasing the copy number of the *CWP2* gene.

The C terminus of Cwp2 functions as a GPI cleavage/attachment signal. Indirect evidence suggests that the Cwp2 protein receives a GPI anchor in the ER and then moves through the protein secretory pathway to the plasma membrane, where it is cleaved from the anchor and covalently attached to carbohydrates in the outer cell wall (50). It is not possible to determine if intracellular, precursor Cwp2 protein has a GPI anchor because there is no assay for the precursor. Attempts to make polyclonal rabbit antibodies against synthetic peptides failed to yield serum that would detect any form of the Cwp2 protein,

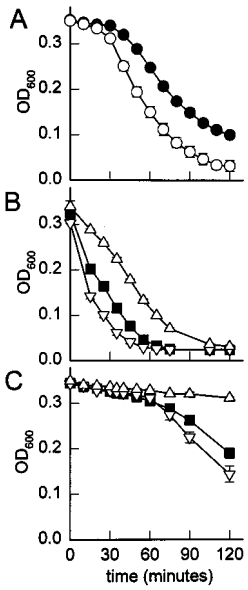

FIG. 2. *CWP2* protects cells from glucanase digestion. Cells were grown without (A and B) or with (C) 25 μM PHS at 30°C overnight to an OD_{600} of 0.6 in PYED-5.0, centrifuged, resuspended in buffer, and digested as described in Materials and Methods. Strains used for the experiments shown in each panel were determined to be growing at the same rate and to be in the mid-log phase of growth (data not shown). The averages and standard deviations from four determinations are shown. Symbols: ●, SJ21R (wild type); ○, MS200 (SJ21R with *cwp2* deleted); ■, 4R3 (*CWP2*); ▽, MS100 (*cwp2-Δ1*); △, MS106 (six copies of *CWP2*).

not a surprising result since the protein is heavily glycosylated and likely to be unreactive with antibodies raised against unglycosylated protein.

As an alternative, we sought to demonstrate that the C terminus of the Cwp2 protein can function as a GPI anchor/cleavage signal. One of the more thoroughly characterized GPI-anchored protein in *S. cerevisiae* is Gas1. Its journey to the plasma membrane begins in the ER, where the most abundant species is a 105-kDa glycosylated, GPI-anchored precursor (11, 48). The precursor is transported by COPII vesicles (15) to the Golgi apparatus, where further glycosylation yields the mature 125-kDa protein.

A hybrid Gas1 protein (GAS-CWP) having its C-terminal cleavage/attachment site and membrane-spanning domain, residues 506 to 537, replaced with the putative C-terminal cleavage/attachment site and membrane-spanning domain of Cwp2, residues 71 to 92, was constructed and produced by using a *CEN* vector in a strain of *S. cerevisiae* deleted for *gas1*. By two criteria, the hybrid GAS-CWP protein functioned like the wild-type protein. First, the *gas1*-deleted strain carrying the hybrid protein grew as fast as a strain carrying wild-type Gas1 protein, whereas the strain grew slowly when it lacked a functional Gas1 protein or when the protein lacked a cleavage/attachment signal (GAS^{stop}, a stop codon at codon 507). Second, Western blot analysis showed that the GAS-CWP hybrid protein was converted from the 105-kDa precursor to the 125-kDa mature species and accumulated in cells. Thus, it behaved like the wild-type Gas1 protein (Fig. 3A; compare lanes 2 and 4). In contrast, cells containing a variant Gas1 protein lacking a cleavage/attachment signal (GAS^{stop}) showed the same low, nonspecific background level of antibody staining (Fig. 3A, lane 5) as did the control cells deleted for *gas1* (Fig. 3A, lane 1). This is the result expected for Gas1 lacking a GPI anchor, since the unanchored protein is excreted into the culture medium (36). These data demonstrate that the C terminus of Cwp2 can act as a GPI cleavage/attachment signal when attached to the Gas1 protein.

We also determined if Cwp2 requires its putative C-terminal GPI cleavage/attachment signal sequence for function and if this sequence can be replaced by the Gas1 cleavage/attachment sequence. The mutant CWP^{stop} allele, producing a truncated protein of 71 amino acids lacking the putative GPI cleavage/attachment signal, and the chimeric *CWP-GAS* allele, producing residues 1 to 71 of Cwp2 fused to the cleavage/attachment site, residues 507 to 537, of Gas1, were tested for function in a *cwp2* deletion strain by measuring glucanase resistance (Fig. 3B). The CWP^{stop} allele gave slightly more glucanase protection than the control cells lacking a functional *CWP2*, gene while the chimeric *CWP-GAS* gene gave slightly more protection than did the CWP^{stop} gene. Neither the truncated nor the chimeric gene protected cells as well as the wild-type or the control gene containing the *Eco*RI site (Fig. 3B). When this experiment was repeated with the *CWP* genes carried in a multicopy (2μm replicon) rather than a single-copy (*CEN*) vector, the same trends were seen except that glucanase resistance was increased (data not shown). These data show that the Gas1 GPI cleavage/attachment sequence is able to partially substitute for the Cwp2 C terminus. Based on the data presented in Fig. 3, we conclude that the Cwp2 protein has a GPI cleavage/attachment sequence at its C terminus and that this sequence is essential for the protein to be incorporated into the cell wall at a normal rate.

Conversion of the 105-kDa Gas1 protein to the 125-kDa protein requires sphingolipids. Because antibodies capable of detecting the Cwp2 protein are not available, it is not possible

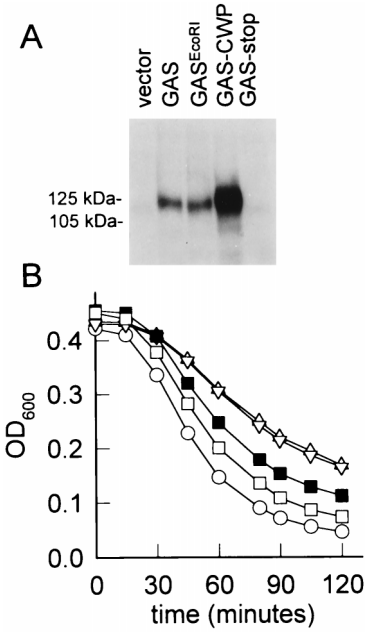

FIG. 3. The Cwp2 protein contains a functional GPI cleavage/attachment signal. The 105- and 125-kDa Gas1 species were detected by Western blot analysis (A) in strain WB2d (*gas1* deleted) transformed with a vector carrying no *CWP2* gene (lane 1), wild-type *GAS1* (lane 2), $GAS1^{EcoRI}$ (lane 3), the *GAS-CWP* chimera having the putative Cwp2 cleavage/attachment signal in place of the normal Gas1 signal sequence (lane 4), and the $GAS1^{stop}$ allele lacking the cleavage/attachment signal (lane 5). The *GAS-CWP* and $GAS1^{stop}$ alleles were made from the GAS^{EcoRI} allele. Panel B shows the results of glucanase digestion of MS200 (*cwp2* deleted) cells transformed with the vector carrying no *CWP2* gene (○), $GAS1^{stop}$ (□), *GAS-CWP* (■), GAS^{EcoRI} (▽), or wild-type *CWP2* (△).

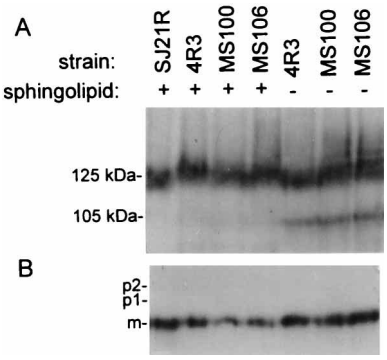

FIG. 4. Increased 105-kDa Gas1 precursor in cells lacking sphingolipids. The steady-state concentrations of the 105-kDa Gas1 precursor and its mature 125-kDa product were determined by Western blot analysis of cell extracts prepared from cells grown to mid-log phase. The blot shown in panel A was probed with anti-Gas1 antibodies, and the antibodies were located by using enhanced chemiluminescence and autoradiography. The blot was stripped and reprobed with anti-CPY antibodies (B). Cells were grown in PYED containing 25 μM PHS (+ sphingolipids) or lacking PHS (− sphingolipids). The mobilities of mature (m) and precursor (p1 and p2) species of CPY are indicated.

to measure the effect of sphingolipids on the rate of Cwp2 protein movement through the secretory pathway. However, antibodies for the Gas1 protein are available, and conversion of the 105-kDa Gas1 precursor, found in the ER, to the 125-kDa mature protein, found in the Golgi apparatus and plasma membrane, has been used to measure the rate of vesicle transport from the ER to the Golgi apparatus. Therefore, we examined the effect of sphingolipids on Gas1 transport. We reasoned that the 105-kDa Gas1 precursor should accumulate in cells lacking sphingolipids if sphingolipids are necessary either for addition of the GPI anchor or for vesicle transport from the ER to the Golgi apparatus. By using polyclonal Gas1 antibodies and Western blot analysis (Fig. 4A), the steady-state concentration of the 105 kD Gas1 species was found to be at least 10-fold higher in cells lacking sphingolipids (lanes 5 to 7) than in cells containing sphingolipids (lanes 1 to 4); the difference could be larger because it is difficult to measure the very low level of precursor in cells containing sphingolipids. Accumulation of the 105-kDa precursor indicates that sphingolipids are necessary for a normal rate of transport of the Gas1 protein.

To determine if the effect of sphingolipids was specific for GPI-anchored proteins, maturation of a non-GPI-anchored protein, CPY, was examined by reprobing the Western blot shown in Fig. 4A with a monoclonal CPY antibody. Only the mature 61-kDa vacuolar form, not the 67-kDa ER or the 69-kDa Golgi (46) form, was detected in cells either lacking or containing sphingolipids (Fig. 4B), indicating that sphingolipids are not required for maturation of this protein.

The rate of precursor conversion to the 125-kDa Gas1 product was measured by using a pulse-chase radiolabeling protocol followed by immunoprecipitation of Gas1. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. About 50% of the radiolabeled Gas1 precursor matures to the 125-kDa species within 5 min in wild-type SJ21R cells, while the same degree of maturation takes about 10 min in 4R3 cells containing sphingolipids (Fig. 5A and B). In 4R3 cells lacking sphingolipids, the rate of maturation is greatly reduced so that even after a 60-min chase period, only about half of the radiolabeled Gas1 protein matures to the 125-kDa species; more than 90% of the radiolabeled protein matures to the 125-kDa species in SJ21R cells, while slightly less, 80%, does so in 4R3 cells containing sphingolipids (Fig. 5A and B).

The rate of maturation of CPY was also determined. About 50% of the precursors species were converted to the mature 61-kDa species within 4 to 5 min in wild-type SJ21R cells, while the time required was slightly less, 8 to 10 min, in 4R3 cells containing sphingolipids (Fig. 5C and D). However, the rates of maturation in 4R3 were identical in cells containing or lacking sphingolipids. By the 60-min time point, almost all of the radiolabeled precursors were converted to the mature species in all cells examined.

We conclude from the data presented in Fig. 4 and 5 that sphingolipids are essential for a normal rate of transport from the ER to the Golgi apparatus of the GPI-anchored Gas1 protein but not for the CPY protein which lacks a GPI anchor.

Accumulation of immature Gas1 following starvation for long-chain base. Further evidence that sphingolipids are necessary for a normal rate of transport of GPI-anchored proteins was obtained by using an alternative experimental approach.

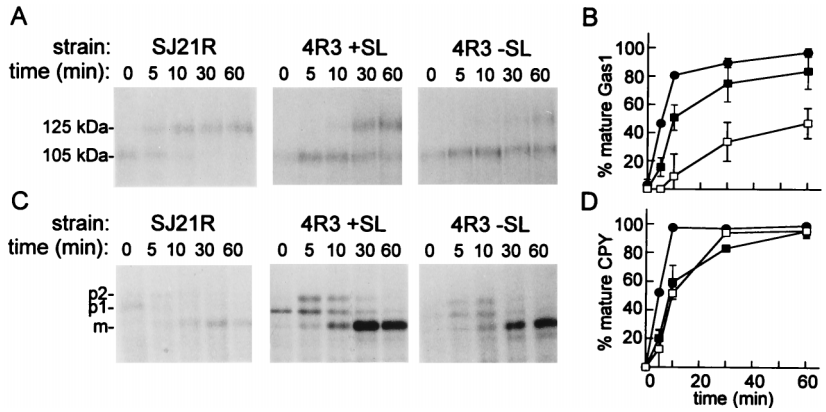

FIG. 5. Sphingolipids are required for a normal rate of maturation of the Gas1 protein. The rate of conversion of the 105-kDa Gas1 precursor to its mature 125-kDa product was determined by using a pulse-chase radiolabeling protocol. At time zero, the incorporation of radiolabeled amino acids into proteins was terminated by dilution with nonradioactive amino acids. At the indicated times, Gas1 protein (A and B) or CPY protein (C and D) was immunoprecipitated and separated by SDS-PAGE, and the gel was autographed. The amount of radioactivity in bands was quantified by scanning the autoradiogram and analyzing the scan with SigmaScan software. The average values plus standard deviations from three experiments are plotted as percentages of radioactive Gas1 protein present as the mature 125-kDa species (B) or percentages of CPY present as the mature (m) 61-kDa species (D). The p1 and p2 CPY precursors are indicated. Symbols: ●, SJ21R (wild type); ■, 4R3 containing sphingolipids; □, 4R3 lacking sphingolipids.

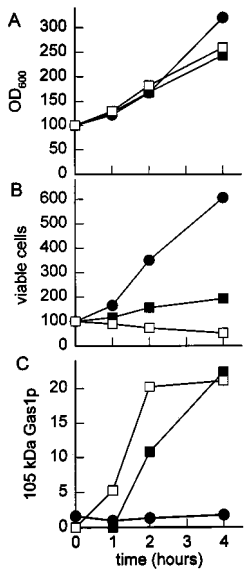

FIG. 6. Cells starved for long-chain base accumulate the 105-kDa Gas1 precursor. Cells were pregrown with PHS, washed, and at time zero resuspended in medium lacking PHS as described in Materials and Methods. Growth was monitored spectrophotometrically (A). Cell viability was measured by plating dilutions of cultures on PYED plates containing 25 μM PHS (B). Amounts of the 105-kDa Gas1 precursor and the mature 125-kDa protein were measured by Western blot analysis (C). The amount of the 105-kDa Gas 1 species present on the blot was quantified as described in Materials and Methods. Data are plotted as a percentage of the time zero value (A and B) or as the precursor species as a percentage of the total Gas1 protein (C). Symbols: ●, SJ21R: ■, 4R3; □, 1Δ4.

Withdrawal of long-chain base from an Lcb$^-$ strain reduces sphingolipid synthesis within 2 h but does not affect synthesis of glycerophospholipids, DNA, and proteins, which continue to be made for several hours. During this time frame, cell mass increases but viability decreases (39). We reasoned that the 105-kDa Gas1 precursor should accumulate when long-chain base is withdrawn from the Lcb$^-$ strain 1Δ4 if sphingolipids are necessary for transport of the Gas1 precursor from the ER to the Golgi apparatus. During the first hour after long-chain base starvation, the OD_{600} of the 1Δ4 culture increased as did that of the wild-type culture (Fig. 6A) and there was a small loss of viability (Fig. 6B). In this same time frame, the 105-kDa Gas1 precursor was already accumulating in starved 1Δ4 cells but not in wild-type cells (SJ21R) or in starved 4R3 cells (Fig. 6C). The 105-kDa protein continued to accumulate in starved 1Δ4 cells for 2 h but did not accumulate in wild-type cells. The protein started to accumulate in 4R3 cells between 1 and 2 h after long-chain base starvation because sphingolipid synthesis had ceased. Thus, data obtained by using this second experimental approach support the hypothesis that sphingolipids are necessary for a normal rate of transport of the 105-kDa Gas1 precursor from the ER to the Golgi apparatus.

The 105-kDa Gas1 precursor accumulates as a GPI-anchored protein. To begin to understand which step in transport requires sphingolipids, we determined whether the 105-kDa Gas1 precursor accumulates with or without a GPI anchor in 4R3 cells lacking sphingolipids. The presence of a GPI anchor on Gas1 was assessed by measuring detergent solubility before and after treatment with PI-PLC. The Gas1 protein is soluble in the detergent Triton X-114 when anchored to GPI, but following treatment of membranes in vitro with PI-PLC to remove the GPI anchor, the protein converts to a water-soluble species (11, 48).

Nearly all of the 105-kDa Gas1 precursor accumulated in 4R3 cells lacking sphingolipids partitions into the detergent phase before treatment of the membrane samples with PI-PLC, but after enzyme treatment, the precursor partitions into the aqueous phase (Fig. 7; compare lanes 6 and 9). This is the behavior expected for a GPI-anchored protein and is what we observed for the GPI-anchored 125-kDa mature Gas1 protein in wild-type and 4R3 cells containing or lacking sphingolipids (Fig. 7). Following PI-PLC treatment, there was a band of about 95 kDa present in the aqueous phase of the 4R3 sample lacking sphingolipids (lane 9). This band probably represents a proteolysis product since it was only seen after overnight incubation with PI-PLC. Upon longer exposure, it was seen also in lanes 7 and 8 (data not shown). Based on these data, we conclude that the 105-kDa Gas1 precursor accumulates in 4R3 cells lacking sphingolipids as a GPI-anchored species. The aqueous phase of samples not treated with PI-PLC shows either a smear or two bands about the size of the 125-kDa Gas1 protein (Fig. 7, lanes 1 to 3). Since these reactants were not observed in all Western blots (data not shown) and since they were sometimes observed in a *gas1* deletion strain (Fig. 3, lane 1), they most likely represent a soluble protein that cross-reacts with our Gas1 antibody.

Since the 105-kDa Gas1 precursor accumulates with a GPI anchor, we conclude that anchor synthesis and attachment do not require sphingolipids: sphingolipids must be necessary for a subsequent step in transport to the Golgi apparatus.

DISCUSSION

We have used suppressor gene analysis to investigate why sphingolipids are necessary for survival of *S. cerevisiae* cells at low pH. This analysis led to the discovery that the *CWP2* gene was a suppressor. So why should the lack of sphingolipids be compensated for at low pH by overproduction of a cell wall mannoprotein? The Cwp2 protein has a putative GPI attachment signal at its C terminus and a putative membrane-spanning domain at its N terminus, suggesting that it is attached to GPI in the ER and transported to the cell surface as a GPI-anchored protein (50). Horvath et al. (23) showed that blockage of sphingolipid synthesis by a drug inhibited transport of the GPI-anchored protein Gas1, and they suggested that sphingolipids enhanced transport of GPI-anchored proteins from the ER to the Golgi apparatus in yeast. Thus, one explanation for decreased survival at low pH is that the rate of transport of GPI-anchored proteins is reduced in cells lacking sphingolipids. As a result, less Cwp2 protein reaches the cell wall, protection against the stress of low pH decreases, and survival is reduced. Multiple copies of *CWP2* increase the rate of Cwp2

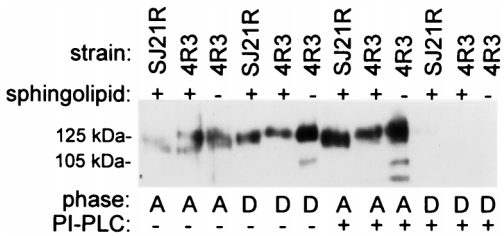

FIG. 7. The 105-kDa Gas1 precursor accumulates with a GPI anchor. The presence of a GPI anchor on the 105-kDa Gas1 precursor was assessed by determining if the protein was soluble in the detergent (lanes D) or aqueous (lanes A) phase before (−) or after (+) treatment of membrane samples with PI-PLC. Cells were grown with (+ sphingolipids) or without (− sphingolipids) 25 μM PHS in the culture medium. Proteins were prepared from cells grown to mid-log phase (OD_{600} of 0.6) and separated by SDS-PAGE. The Gas1 protein was detected by Western blot analysis.

protein transport, resulting in a more protective cell wall and increased survival. Our data (Fig. 1 to 5) support this explanation and lead us to conclude that sphingolipids are necessary for a normal rate of transport of GPI-anchored proteins from the ER to the Golgi apparatus. This conclusion is further supported by experiments showing a block in transport of the GPI-anchored Gas1 protein following blockage of sphingolipid synthesis by starving an Lcb^- auxotroph for PHS (Fig. 6). The effect of sphingolipids is specific to GPI-anchored proteins since no effect on a transmembrane, vacuolar protein, CPY, was observed (Fig. 4 and 5). Our data thus confirm and extend the work of Horvath et al. (23).

An attribute of the suppressor gene approach is its ability to identify genes that overcome growth defects. Because this experimental approach produces a gain of function, we feel confident that a direct and physiologically relevant function of sphingolipids has been identified. In contrast, the drug inhibition approach of Horvath et al. (23) and our approach of blocking sphingolipid synthesis by withholding PHS from an Lcb^- auxotroph involve loss of function and could reflect effects of reduced sphingolipid synthesis on an unidentified essential cellular process rather than a direct effect on vesicle transport. Each of the three approaches has other attributes and deficiencies, but when the results of all three approaches are combined, they provide strong support for the hypothesis that sphingolipids are essential for a normal rate of transport of GPI-anchored proteins from the ER to the Golgi apparatus in *S. cerevisiae.*

How might sphingolipids facilitate transport of GPI-anchored proteins? One possibility is that sphingolipids initiate or modulate some step in transport of vesicles containing GPI-anchored proteins. The concentration of ceramide is likely to be at its highest in the ER, as this is its site of synthesis (41). Ceramide could thus serve to mark the ER membrane and thereby play a key role in vesicle formation. Gas1p is known to be transported from the ER to the Golgi apparatus in vesicles containing a COPII protein coat (15). Since the COPII budding process can be reproduced in vitro by using membranes and either purified coat proteins or a cytosolic extract (3, 4, 52), it may be possible to begin to identify which step and protein(s) in vesicle transport require sphingolipids.

An alternative hypothesis to explain the role of sphingolipids, termed the clustering hypothesis by Horvath et al. (23), is based on studies in animals cells showing clustering of GPI-anchored proteins with glycosphingolipids and cholesterol to form microdomains that originate in the *trans*-Golgi network and move to specific plasma membrane targets by vesicle transport (8, 45). Based on the propensity of GPI-anchored proteins to cluster with sphingolipids, it may be that at least in *S. cerevisiae*, there is clustering of sphingolipids and GPI-anchored proteins in the ER, with the clusters playing a role in formation of or partitioning into COPII transport vesicles.

As far as we are aware, the requirement of sphingolipids for transport of GPI-anchored proteins from the ER to Golgi apparatus has not been directly examined in higher eucaryotes, and so detergent-insoluble glycosphingolipid-enriched complexes containing GPI-anchored proteins may form in the ER to facilitate vesicle transport to the Golgi apparatus. However, one argument against this possibility is the difference in the sites of synthesis of complex sphingolipids in yeasts and higher eucaryotes. Ceramide is the only sphingolipid thought to be present in high quantity in the ER of higher eucaryotes (49). It is transported to the Golgi apparatus, where the complex sphingolipids, sphingomyelin and glycosphingolipids, found in microdomains with GPI-anchored proteins, are synthesized (49). Studies with mutants of *S. cerevisiae* blocked in the protein secretory pathway suggest that, in addition to ceramide, the complex sphingolipid IPC is present in the ER, and it might form microdomains with GPI-anchored proteins as a prerequisite for vesicle transport (41). However, caution should be exercised in extrapolating data on the location of IPC in *sec* mutants to the location in wild-type cells, particularly since the location of the enzyme, IPC synthase, for making IPC is not known, nor has its trafficking between membranes been examined.

One other potentially significant difference between ceramides and sphingolipids in yeasts and higher eucaryotes is the presence in all yeast ceramides and sphingolipids of a very long chain fatty acid, primarily C_{26} (29). It may have a propensity to form microdomains with GPI-anchored proteins in the ER and thus play a fundamental role in vesicle transport.

In *S. cerevisiae*, the diacylglycerol moiety of many GPI anchors is replaced by a ceramide moiety after transport from the ER (12). Thus, one explanation for a reduced amount of the Cwp2 protein in the wall of cells lacking sphingolipids (Fig. 2) is that the GPI anchor is not remodeled with ceramide, which reduces either its rate of delivery to the plasma membrane or a step in the process that attaches Cwp2p to the glucan layer. This possibility cannot be ruled out at the moment because it is not possible to isolate and analyze the GPI anchor on intermediates in Cwp2 protein maturation. Also, this possibility cannot explain the reduced rate of Gas1 transport from the ER to the Golgi apparatus in the absence of sphingolipids because the GPI anchor on Gas1 does not contain ceramide (18).

Enhanced survival of cells lacking sphingolipids at low pH by multiple copies of the *CWP2* gene indicates (Fig. 1B) that the Cwp2 protein is limiting for a process that protects cells against protons. The Cwp2 protein may directly shield the cell from protons since it is abundant and necessary for forming a thick outer layer on yeast cells (50). Alternatively or in addition, the protective role of Cwp2 may be indirect and serve to maintain the integrity of the cell wall and plasma membrane.

The protective role of the Cwp2 protein is apparent also when sphingolipids are present, since a strain with multiple copies of the *CWP2* gene survives low-pH treatment better than cells with one copy of the gene, which in turn survives better than cells with no *CWP2* gene (Fig. 1A). The ability of the *cwp2* deletion strain MS100 to survive fairly well at low pH when sphingolipids are present is most likely due to other cell wall proteins such as Cwp1, Tip1, and Srp1 compensating for the lack of Cwp2 (50). Cwp1, Tip1, and Srp1 are thought to be GPI anchored during transport to the plasma membrane. In the absence of sphingolipids, their rate of transport is likely to be reduced, and so too the cell's defense against protons and other stresses.

Since the discovery of the novel suppressor lipids, C_{26} fatty acyl-containing inositol glycerophospholipids, made by strain 4R3 grown without a long-chain base, it has been unclear how such lipids compensate for the lack of sphingolipids and promote growth (30). The data presented here suggest that one compensatory role for the suppressor lipids is to restore ER-to-Golgi transport of GPI-anchored proteins to a level supportive of growth during nonstressful conditions (38). In addition, they may be used in place of ceramide to remodel GPI-anchored protein, one or more of which is necessary for growth.

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