Sphingolipids Are Required for the Stable Membrane Association of Glycosylphosphatidylinositol-anchored Proteins in Yeast*

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Ongoing sphingolipid synthesis is specifically required in vivo for the endoplasmic reticulum (ER) to Golgi transport of glycosylphosphatidylinositol (GPI)-anchored proteins. However, the sphingolipid intermediates that are required for transport nor their role(s) have been identified. Using stereoisomers of dihydrospHINGosine, together with specific inhibitors and a mutant defective for sphingolipid synthesis, we now show that ceramides and/or inositol sphingolipids are indispensable for GPI-anchored protein transport. Furthermore, in the absence of sphingolipid synthesis, a significant fraction of GPI-anchored proteins is no longer associated tightly with the ER membrane. The loose membrane association is neither because of the lack of a GPI-anchor nor because of prolonged ER retention of GPI-anchored proteins. These results indicate that ceramides and/or inositol sphingolipids are required to stabilize the association of GPI-anchored proteins with membranes. They could act either by direct involvement as membrane components or as substrates for the remodeling of GPI lipid moieties.

Sphingolipids are a major lipid component in eukaryotic cells. They are not only membrane components, but they also have important functions in regulation of cellular events (1). In Saccharomyces cerevisiae, sphingolipids are involved in stress responses, calcium homeostasis, regulation of cell growth, cell cycle control, and membrane traffic (1).

Glycosylphosphatidylinositol (GPI)-anchored proteins are a group of proteins that are expressed on the surface of eukaryotic cells. They are modified post-translationally with a glycosylphosphorylceramide; ER, endoplasmic reticulum; PI, phosphatidylinositol; PLC, phospholipase C; C26, 26 carbon containing.

(2–5). One of these is ongoing sphingolipid synthesis. This requirement has been identified by the observation of impaired trafficking of these proteins in several mutants defective at different steps of sphingolipid biosynthesis. These mutants lag1 lac1, which are defective in acyl-CoA-dependent ceramide synthesis (6–8), and fen1 (elo2) and sur4 (elo3) mutants, which are defective in the synthesis of C26 fatty acyl-CoA, a precursor of ceramides and sphingolipids in yeast (9, 10). These results suggest strongly that certain sphingolipids are required specifically for the efficient transport of GPI-anchored proteins, but so far the relevant sphingolipids have not been identified.

GPI-anchored proteins also are transported from the ER to the Golgi apparatus in distinct vesicles from other secretory proteins (11). The sorting of GPI-anchored proteins is dependent on the proper functions of the Rab GTPase Ypt1p and the tethering factors Uso1p and COG complex (Sec34/35p) (12). In addition to these protein factors, sphingolipid/sterol-enriched detergent-insoluble microdomains, also known as lipid rafts, may also function in the sorting of GPI-anchored proteins. Rafts may function in several cellular events such as signal transduction, protein sorting, and lipid traffic (13). In yeast, raft-like structures have been proposed to play several roles in protein sorting (14, 15). It was shown that GPI-anchored proteins are detergent-insoluble in the ER, and thus, rafts have been proposed to have a sorting function there (14). Defining the precise function of sphingolipids in GPI-anchored protein transport in yeast may unravel the sorting mechanism of GPI-anchored proteins in the ER and the significance of raft structure in their transport.

In this study, we show that ceramide and/or inositol sphingolipids are indispensable for GPI-anchored protein transport to the Golgi compartment. Interestingly, we found that in the absence of ceramide, a significant fraction of GPI-anchored proteins is no longer associated tightly with the ER membrane but behaves like peripheral membrane protein. This loose membrane association was neither because of the lack of a GPI-anchor attachment nor to the retention of the GPI-anchored proteins in the ER. The lack of tight membrane association is likely to be one of the reasons for the inefficient transport of GPI-anchored proteins to the Golgi.

MATERIALS AND METHODS

Strains, Plasmids, Media, and Reagents—The strains used in this study are listed in Table I. RH5465 was obtained by crossing RH3804 with RH3804. Yeast cells were grown overnight in semi-synthetic medium, SDYE (2). To express wild-type and mutant Gas1p (L526R) in gas1 knock-out strains, we used plasmids YEplac195-GAS1 and PC-NYG-L526R (16), respectively. To construct YEplac195-GAS1, a 2.4-kbp fragment of the GAS1 gene including the promoter region was
amplified by PCR with the primers 5’-GTCGCGGATCCGCTTCATATGAAGGTCAGGGCCGcTGGCTA and 5’-GTCGCGGGACCTCACTGATATTATGAGAAAGTACATA. The fragment was subcloned into YEplac195 (2 µm; URA3) using SphiI and SstI sites in the fragment and the vector. To induce Gap1p expression we precultured strains in SUD (20 g/liter glucose, and 1.0 g/liter urea plus required supplements) overnight before starting cultures in SDYE. Stereospecifics of dihydroxyphosphinosine (DHS) and phytophosphinosine (PHS) were purchased from Metrex (Pleasant Gap, PA) and Sigma, respectively, and were dissolved in 100% ethanol at 10 mM as stock solutions.

**Sphingolipid Analysis—In vivo** [3H]myo-inositol labeling was performed as described previously (17) with slight modifications. Briefly, cells were grown overnight in SDYE at 24 °C for 3 h. Triton X-114 phase separation was done in the presence of 0.125 mg/ml bovine serum albumin and ovalbumin in the medium (16). Membrane Association Assay—To follow the membrane association of transport intermediates, we pulse-labeled cells for 6 min and chased for 15 min as described above. The radiolabeled cells were suspended in TEPI and lysed with glass beads. After removal of insoluble material by centrifugation at 2,500 × g for 5 min, the crude cell extracts were divided into three fractions. One was mixed with an equal amount of TEPI, one was mixed with 0.2 M Na2CO3, pH 12, in TEPI (to give final pH of 11), and one was mixed with 2% (v/v) Triton X-100 in TEPI and incubated on ice for 30 min. Soluble and pellet fractions were prepared by centrifugation at 150,000 × g for 30 min at 4 °C. The soluble fractions from 0.1 M Na2CO3 treatment were precipitated with 10% trichloroacetic acid, and other fractions were solubilized directly at 55 °C for 5 min by addition of SDS to 1%. All were processed for immunoprecipitation using the corresponding rabbit antiserum and protein A-Sepharose.

**RESULTS**

**Substrate Specificities of Ceramide Synthase and IPC Synthase in Yeast**—To better define the role of sphingolipids in GPI-anchored protein transport from the ER to the Golgi compartment, we first investigated the structural requirements for sphingolipid synthesis using stereoisomers of the ceramide precursor, DHS, whose structures are shown in Fig. 1A. d-erythro-DHS is the naturally occurring form. To analyze sphingolipid synthesis, we preincubated wild-type and lcb1-100 mutant cells at 37 °C for 15 min in the presence or absence of each DHS isomer and then labeled sphingolipid by adding [3H]myo-inositol for 60 min. As described previously (17), lcb1-100 has an extremely low level of sphingolipid synthesis (Fig. 1B, lane 4) at 37 °C when compared with wild-type cells (lane 2). Addition of d-erythro-DHS restored the biosynthesis of all sphingolipids in lcb1-100 mutant cells at 37 °C (lane 6). d- and l-erythro-DHS restored sphingolipid biosynthesis in lcb1-100 mutant cells to the same extent as d-erythro-DHS (lanes 8 versus lane 6), but the other two stereoisomers, l- and d-erythro-DHS, could not restore sphingolipid synthesis (lanes 10 and 12).

Next, to determine the stereospecificity of ceramide synthesis, we measured ceramide synthesis activity using microsomal membranes in the presence of d-erythro and l-erythro forms of [3H] DHS, as described previously (18). We found that dihydro-ceramide was synthesized from the d-erythro but not the l-erythro form of [3H] DHS (Fig. 1C), suggesting that ceramide...
Identification of Indispensable Sphingolipids for GPI-anchored Protein Transport completely (Fig. 2A). These results suggest that ceramide and/or inositol sphingolipids are required for transport. To confirm this, we studied the effects of inhibitors specific for syntheses of ceramide and inositolphosphorylceramide (IPC). In the presence of the ceramide synthesis inhibitor australifungin (21), the restoration of GPI-anchored protein transport completely (Fig. 2A). This suggests that ceramide and/or inositol sphingolipids play a role in transport. The complete inhibition by both inhibitors could be because of incomplete inhibition of ceramide and IPC syntheses under these conditions (high DHS concentration) or could be because of a partial restoration by DHS. To provide additional evidence for a requirement for ceramide synthesis, we tested transport efficiency under the condition when ceramide synthesis is eliminated almost completely by other means. The mutant lcb3, ysr3 double knockout mutants, which are defective for dephosphorylation of phosphorylated sphingoid base, combined with lcb1-100, produce very little ceramide and inositol sphingolipid from exogenous DHS (17, 23). We compared the concentration of DHS that efficiently restored transport defect in these cells versus that needed for the lcb1-100 mutant (Fig. 2B). For lcb1-100 cells, 10 μM d-erythro-DHS restored GPI-anchored protein transport considerably. In contrast, in lcb1/3, ysr3 cells, transport was not.

synthesis is stereospecific. We observed that the synthesis of dihydroceramide was fumonisin B1-sensitive, confirming we are measuring acyl-CoA-dependent ceramide synthesis activity (20).

Identification of Indispensable Sphingolipids for GPI-anchored Protein Transport from the ER to the Golgi Compartment—Next, we studied GPI-anchored protein transport (Fig. 2A) under the same conditions we used to assay sphingolipid biosynthesis. After a 15-min preincubation of lcb1-100 mutant cells at 37 °C in the presence or absence of individual DHS stereoisomers, we labeled cells with [35S]-methionine, and chased for the indicated period of time. The percentage of mature Gas1p is shown. B, comparison of effects of d-erythro-DHS for the restoration of the Gas1p transport defect in lcb1-100 and lcb1/3/ysr3 cells. Strains RH3809 (lcb1-100) and RH3859 (lcb1/3/ysr3) were assayed for ER to Golgi transport of Gas1p in the presence of different concentrations of exogenous d-erythro-DHS. C, restoration of the Gas1p transport defect by d-erythro-DHS or d-erythro-PCS in sphingoid base metabolism mutants. Strains RH4355 (lcb1/45) and RH4363 (lcb1/sur2) were assayed for the ER to Golgi transport of Gas1p in the presence or absence of exogenous d-erythro-DHS.

Fig. 2. Identification of the essential sphingolipids for ER to Golgi GPI-anchored protein transport. A, restoration of the Gas1p transport defect in lcb1-100 cells by stereoisomers of DHS. Mutant lcb1-100 (RH3809) cells were preincubated at 37 °C for 15 min in the absence or presence of individual DHS stereoisomers (50 μM) and ceramide synthesis inhibitor australifungin (5 μg/ml) or IPC synthesis inhibitor aureobasidin A (20 μg/ml), labeled with [3H]-methionine, and chased for the indicated period of time. The percentage of mature Gas1p is shown. B, comparison of effects of d-erythro-DHS for the restoration of the Gas1p transport defect in lcb1-100 and lcb1/3/ysr3 cells. Strains RH3809 (lcb1-100) and RH3859 (lcb1/3/ysr3) were assayed for ER to Golgi transport of Gas1p in the presence of different concentrations of exogenous d-erythro-DHS. C, restoration of the Gas1p transport defect by d-erythro-DHS or d-erythro-PCS in sphingoid base metabolism mutants. Strains RH4355 (lcb1/45) and RH4363 (lcb1/sur2) were assayed for the ER to Golgi transport of Gas1p in the presence or absence of exogenous d-erythro-DHS.

Fig. 1. Stereospecificity of sphingolipid biosynthesis in yeast. A, structures of dihydroinososine stereoisomers are shown and are designated using the R/S nomenclature. d-erythro- and l-erythro-DHS are enantiomers, as are d-threo- and l-threo-DHS; the d-threo- and l-erythro-DHS are diastereomers. The threo forms differ from the erythro forms in that C2 and C3 are in the cis configuration in the threo forms but trans in the erythro forms. B, in vivo [3H]myo-inositol labeling to measure sphingolipid synthesis. Wild-type (RH1800) or lcb1-100 mutant (RH3809) cells were preincubated at 37 °C for 15 min in the absence or presence of each DHS stereoisomer (50 μM) and labeled with [3H]myo-inositol for 60 min. Incorporation of [3H]myo-inositol into the total lipid fraction was quantified, and equal amounts of radioactive lipids were treated with mild base to identify sphingolipids (even-numbered lanes). MIPC, mannosylated inositolphosphorylceramide; M(IP)2C, mannosylated diinositolphosphorylceramide. C, in vitro ceramide synthesis assay using d-erythro and l-erythro form of [3H]-DHS. Microsomal membranes (200 μg) and cytosol (100 μg) were incubated with [3H]DHS for 2 h at 24 °C in the presence or absence of Fumonisin B1 (100 μM). Lipids were extracted and analyzed by TLC using solvent system II. DH-ceramide, dihydroceramide.
restored significantly even at 50 μM v-erythro-DHS. From these results, we conclude that ceramide and/or inositol sphingolipid synthesis is necessary for GPI-anchored protein transport.

To address the possibility that other DHS derivatives, specifically phosphorylated sphingolipids or PHS, might be required for GPI-anchored protein transport, we used two other mutant strains. Mutant lcb4, lcb5 double knock-out cells also carrying the lcb1-100 allele cannot produce DHS-1P from exogenous v-erythro-DHS (17, 24), and sur2-disrupted cells, combined with the lcb1-100 allele, are defective for hydroxylation of exogenous DHS to form PHS (17, 25). GPI-anchored protein transport was clearly restored by exogenous v-erythro-DHS in lcb1/4/5 mutant cells and by both v-erythro form of DHS or PHS in lcb1/sur2 mutant cells (Fig. 2C). These results show that phosphorylation of sphingolipids and their interconversion between DHS and PHS are not required for GPI-anchored protein transport.

Loose Membrane Association of GPI-anchored Proteins in lcb1-100 Mutant Cells—To understand the role of sphingolipids in GPI-anchored protein transport, we attempted to use an in vitro assay that measures the efficiency of packaging of proteins into ER-derived vesicles, sorting from other secretory proteins, and the efficiency of delivery of cargo proteins to the Golgi structure (11). During the course of this study, we noticed that a significant fraction of GPI-anchored proteins was released specifically from lcb1-100 mutant membranes to the soluble fraction. This release was cytosol-independent (data not shown) and was enhanced at elevated temperature (30 °C) and by addition of an energy source (ATP and GTP; data not shown). Therefore, we investigated the membrane association of GPI-anchored proteins in lcb1/100 cells using conventional fractionation methods. We preincubated mutant or wild-type cells for 15 min at 37 °C, pulse-labeled for 6 min, and chased for 15 min. Then, we prepared crude cell extracts and assessed membrane association of two GPI-anchored proteins under three different conditions, buffer alone, high pH, and detergent (Fig. 3A). In lcb1-100 mutant cells, but not in wild-type cells, a significant fraction of Gas1p was more loosely associated with membranes even under the buffer conditions (39% versus 7.7%). MoreGas1p was released from the membrane by Na2CO3 pH 11, treatment (54% versus 7.2%). Gas1p has been proposed to associate with detergent-insoluble structures in the ER (26). In accordance with these findings, Gas1p was solubilized poorly by 1% Triton X-100 from wild-type cells. However, Gas1p was solubilized almost completely from lcb1-100 mutant cells (91% versus 53%). We also observed a similar weak membrane association of another GPI-anchored protein, Yps1p, in lcb1-100 cells. In contrast to wild-type cells, most of Yps1p was in the ER form after a 15-min chase in lcb1-100 cells, confirming that the general maturation delay of GPI-anchored proteins in lcb1-100 cells. We also noticed that the small amount of mature forms of Gas1p and Yps1p seen in lcb1-100 cells were associated more tightly with membranes than the ER forms. As controls for these experiments, we examined membrane association of α-COP (peripheral protein) and Gap1p (integral membrane protein). Both proteins behaved as expected in these cell types. From these results, we conclude that the binding of GPI-anchored proteins to the ER membrane is weakened severely in lcb1-100 mutant cells.

To rule out the possibility that prolonged ER retention is the cause of the weakened membrane association of GPI-anchored proteins in lcb1-100 mutant cells, we assayed membrane association in sec18 mutant cells, which shows a block in all ER to Golgi protein transport. The ER forms of Gas1p and Yps1p accumulated in sec18 mutant cells were not released like those in the lcb1-100 mutant. The results confirmed that the weak membrane association in lcb1-100 cells was not because of its retention in the ER.

Next, to investigate the possibility that loose membrane association might be because of inefficient GPI-anchor assembly or addition in lcb1-100 cells, we examined the partitioning behavior of Gas1p using Triton X-114 (16). We labeled cells as in Fig. 3A, prepared cell extracts, solubilized extracts in Triton X-114 at 4 °C, and subjected them to phase partitioning at 32 °C. Fig. 3B shows that Gas1p from wild-type and lcb1-100 cells partitions into the Triton X-114 detergent phase with the same efficiency (left columns). In addition, the Gas1p found in the detergent phase could be shifted completely to the aqueous phase by treatment with phosphatidylinositol-specific phospholipase C to remove the diacylglycerol moiety of GPI-anchor

FIG. 3. Membrane association of GPI-anchored proteins is severely weakened in lcb1-100 mutant cells. A. Membrane association of GPI-anchored proteins was studied in wild-type (RH2874), lcb1-100 (RH3804), and sec18-20 (RH5465) cells. The cells were preincubated for 15 min at 37 °C, labeled for 6 min, and chased for 15 min. Crude extracts were incubated under three conditions (buffer, TEPI, CO32-1%, 0.1 M Na2CO3, pH 11, in TEPI; TX-100, 1% Triton X-100 in TEPI). After separation by ultracentrifugation, the soluble (S) and the pellet (P) fractions were processed for immunoprecipitation using individual rabbit antisera. B. GPI-anchor attachment was not affected in lcb1-100 cells. The same crude extracts as for A were solubilized with Triton X-114 at 1% final concentration. After partitioning into detergent and aqueous phases, the detergent phase was incubated in the presence or absence of PI-PLC. Phases were re-extracted and processed for Gas1p immunoprecipitation. Unanchored Gas1p segregated into the primary aqueous phase, whereas anchored Gas1p partitioned into the primary detergent phase and shifted into the aqueous phase after phospholipase C treatment. The total amount of Gas1p quantified in each partition was set to 100. A1 and A2, first aqueous and detergent phases, respectively (left panels); A2 and D2, aqueous and detergent phases from D1 after mock (middle panels) and PI-PLC (right panel) treatment, respectively. C, membrane association of the prenylated protein Ypt1p was studied in wild-type (RH1638) and lcb1-100 (RH5241) cells as A.
versible in \textit{lcb1} Mutant Cells—Therefore, most of the \textit{Gas1p} in \textit{lcb1} cells seems unable to reach the Golgi compartment and instead was degraded gradually after a long chase time. Fig. 3C shows that membrane association of \textit{Ypt1p} is similar in wild-type and \textit{lcb1-100} cells, suggesting that the weak membrane association is specific for \textit{GPI-anchored proteins}.

The Soluble Form of \textit{Gas1p} Shows Slow Maturation in Wild-type Cells—As exogenous DHS restores \textit{GPI-anchored protein} transport we checked whether it also restored membrane association of \textit{GPI-anchored proteins}. \textit{\textit{D-erythro-DHS}} clearly restored membrane association, as well as maturation, to a wild-type level. On the other hand, \textit{\textit{l-erythro-DHS}}, which was not incorporated into ceramide, did not restore membrane association of \textit{Gas1p} (data not shown). These results lead to the conclusion that ceramide and/or inositol siphingolipid are necessary for stable membrane association of \textit{GPI-anchored proteins}. These results also suggest that the weak membrane association could be the reason for the transport defect in \textit{lcb1-100} cells.

To test whether weak membrane association of \textit{Gas1p} could cause the transport delay of \textit{Gas1p} in wild-type cells, we used a mutant \textit{Gas1p} containing a point mutation (L526R) in the hydrophobic stretch of the carboxy-terminal sequence that is required for \textit{GPI-anchor} addition. This mutant \textit{Gas1p} (L526R) is no longer \textit{GPI-anchored} and is released into the medium as a soluble mature form (16). A large fraction of the mutant protein behaves as a soluble luminal protein, and the remaining membrane-associated protein can be extracted completely from the membrane by carbonate treatment (Fig. 4A). The behavior of this mutant \textit{Gas1p} is similar in wild-type and \textit{lcb1-100} cells. In a pulse-chase experiment (Fig. 4B), we observed the ER form of mutant \textit{Gas1p} (L526R) in wild-type cells even after 60 min of chase, although a significant proportion of the protein was lost (lower left). Wild-type \textit{Gas1p} was matured almost fully in wild-type cells by this time point (upper left). This result demonstrates that a soluble form of \textit{Gas1p} is matured inefficiently even in wild-type cells. The same result was observed in \textit{lcb1-100} cells (lower right). These results suggest that proper membrane association may be necessary for efficient maturation of \textit{Gas1p}.

Because the mutant \textit{Gas1p} (L526R) is secreted into the medium as a mature form (16), it is possible that the released \textit{Gas1p} in \textit{lcb1-100} cells may also be secreted into the medium as a mature form and escaped our detection. Therefore, we assayed for the secretion \textit{Gas1p} into the medium after a long period of chase. Wild-type \textit{Gas1p} in \textit{lcb1-100} cells was neither matured nor released into medium after 1 and 2 h (Fig. 4C, upper right). In contrast, a significant amount of \textit{Gas1p} (L526R) was released as a mature form into the medium, consistent with the decrease of intracellular \textit{Gas1p} (lower panels). The secreted product from both strains showed a normal apparent molecular weight, demonstrating that there is no general defect in Golgi modifications of \textit{Gas1p} in \textit{lcb1-100} cells. Therefore, most of the \textit{Gas1p} in \textit{lcb1-100} cells seems unable to reach the Golgi compartment and instead was degraded gradually after a long chase time.

The \textit{GPI-anchored Protein Transport Defect Is Partially Reversible in \textit{lcb1} Mutant Cells}—As mentioned above, exogenous \textit{\textit{D-erythro-DHS}} restored \textit{GPI-anchored protein} transport fully in \textit{lcb1-100} mutant cells if added from the beginning of preincubation. More than 95% of \textit{Gas1p} was matured after 90 min (Fig. 5A, lane 4). In contrast, if we added DHS after a 15-min chase, we observed a clear but only partial restoration of transport (lane 6, 57%). The simplest explanation for the partial restoration is that once \textit{Gas1p} molecules are released from the

Fig. 4. A soluble \textit{Gas1p} mutant shows slow maturation in wild-type cells. A, membrane association of mutant \textit{Gas1p} protein (L526R) was studied in wild-type (RH1638) and \textit{lcb1-100} (RH5241) cells as in Fig. 3B, maturation of the wild-type (\textit{wt}) and mutant \textit{Gas1p} proteins was examined by pulse-chase experiments. C, after the different chase periods, cells were separated, and the protein in the media was precipitated by addition of trichloroacetic acid to 10%. \textit{Gas1p} in both fractions were analyzed by immunoprecipitation. For the estimation of the amount of \textit{Gas1p} inside of the cells and the medium, the amount of \textit{Gas1p} recovered without chase was set to 100.

Fig. 5. Membrane association of \textit{Gas1p} after long chase. Mutant \textit{lcb1-100} cells (RH5241) expressing wild-type \textit{Gas1p} were preincubated in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3 and 4) of 50 \(\mu\text{M}\) DHS for 15 min at 37 °C, labeled for 6 min, and chased for 15 min and then \textit{\textit{D-erythro-DHS}} was added to 50 \(\mu\text{M}\) (lane 6) and incubated for additional time. A, pulse-chase experiments. B, membrane association of \textit{Gas1p} after 15 min (upper panel) and 90 min (lower panel) in the total absence of DHS (left column) and in the presence of DHS added after a 15-min chase (right column). In this experiment to suppress synthesis of endogenous sphingoid base completely, serine palmitoyltransferase inhibitor, myriocin (5 \(\mu\text{g/ml}\)), was added from the beginning of the preincubation.
ER membrane, they can no longer be associated efficiently with membrane nor transported to the Golgi for maturation. To test this, we studied the membrane association of Gas1p before and after adding DHS (same conditions as for lanes 5 and 6 in Fig. 5A). Unexpectedly, the amount of released ER form of Gas1p decreased by chasing for another 75 min after adding DHS (Fig. 5B, right column). This result shows that membrane association, as well as the Gas1p maturation defect, is reversible and can be restored. We also checked for membrane association of Gas1p after a 90-min chase in the absence of DHS (left column). Only a small fraction of Gas1p was matured after 90 min. As a result we found that all of the Gas1p that was transported to the Golgi compartment in lcb1-100 mutant was membrane-associated, whereas the ER form was always in the equilibrium between membrane-associated and -soluble forms.

**DISCUSSION**

The major finding of this study is that ceramide and/or inositol sphingolipid synthesis is required for ER to Golgi GPI-anchored protein transport in yeast. We also observed that in lcb1-100 mutant cells, GPI-anchored proteins do not show stable membrane association but rather behave like peripheral membrane proteins. The stable membrane association is dependent on ceramide and/or inositol sphingolipid synthesis. The lack of tight membrane association in the absence of sphingolipid synthesis could be one of the reasons why GPI-anchored proteins are not transported efficiently to the Golgi apparatus.

To identify which sphingolipids are required for ER to Golgi transport of GPI-anchored proteins in yeast, we first determined the substrate specificity for ceramide and sphingolipid synthesis. Our study showed that two stereoisomers of DHS, d-erythro and l-threo, but not l-erythro, are incorporated into ceramide in yeast. The stereospecificity for ceramide synthesis in yeast is similar to that found recently for mammalian cells (19). Both stereoisomers of ceramide that could be made were used for inositol sphingolipid synthesis. We found that only the DHS stereoisomers that could be incorporated into ceramide and inositol sphingolipids restored GPI-anchored protein transport in lcb1-100 cells at 37 °C. The endocytic defect in lcb1-100 mutant cells at 37 °C was restored significantly by all four stereoisomers of DHS, suggesting that all stereoisomers can be taken up and are active. In addition, both ceramide and IPC synthesis inhibitors reduced the restoration of GPI-anchored protein transport by d-erythro-DHS in lcb1-100 cells significantly. Finally, GPI-anchored protein transport was not restored in mutant cells that synthesize almost no ceramide even in the presence of DHS. Taken together, these results demonstrate that ceramide and sphingolipids are essential for GPI-anchored protein transport.

This conclusion is consistent with results of studies showing that GPI-anchored protein transport is affected in mutant cells defective at several steps of sphingolipid metabolism. It was shown previously that a lag1 lac1 double knock-out mutant (6–8), and fen1 and sur4 mutant cells (9, 10) are also defective for GPI-anchored protein transport to the Golgi compartment. These studies suggested that ceramide and sphingolipids might be important for GPI-anchored protein transport. However, all these mutants accumulate sphingolipid intermediates such as PHS and synthesize unknown lipids (6–8), which could confer negative effects on GPI-anchored protein transport. In addition, the effect of these mutations on GPI-anchored protein transport was much weaker than that found for the lcb1-100 cells (6, 10). Because exogenous DHS could restore the conditional and severe defect in lcb1-100 cells completely, it was an ideal system to identify required sphingolipids. DHS itself also seems to be important for membrane trafficking steps, like the internalization step of endocytosis, where it is important in regulating protein kinases (17, 28, 29). Our results show clearly that DHS alone is not sufficient for GPI-anchored protein transport.

One of the significant consequences of the lack of ceramide and sphingolipid synthesis is the incomplete membrane association of the ER form of GPI-anchored proteins. This is not because of a lack of GPI anchor attachment. It was reported recently that ceramide synthase, mediated by Lag1p and Lac1p, is localized in ER membranes (6–8) and that IPC synthase, mediated by Aur1p, is localized to the Golgi structure (30). This makes it likely that ceramide, rather than inositol sphingolipids, is responsible for the ER membrane association of GPI-anchored proteins, although we cannot rule out that inositol sphingolipids can be transported from the Golgi apparatus back to the ER.

Based on the observation that GPI-anchored proteins behave like peripheral membrane proteins in lcb1-100 cells, we studied the maturation efficiency of a soluble Gas1p mutant (LS268R) in wild-type cells to test whether membrane association is important for the efficient transport. The soluble form of Gas1p showed delayed maturation in wild-type cells clearly, suggesting that membrane association is an important factor for transport. However, it could also be that the transport delay is because of the lack of a GPI anchor on this mutant Gas1p, because the anchor may possibly function as an exit signal from the ER (31). We also found that a small amount of the mutant Gas1p was matured and secreted into medium, in contrast to the loosely associated GPI-anchored Gas1p in lcb1-100 mutant cells. These differences suggest that there are additional reasons for the maturation delay of GPI-anchored proteins in the absence of tight membrane association in lcb1-100 cells. One possibility is that the hydrophobic part of the GPI anchor of the released proteins may be recognized by the quality control apparatus and serve to retain these proteins in the ER. Alternatively, the hydrophobic anchor could cause formation of micelle-like structures that could be excluded from ER-derived vesicles because of their size.

We found that whenever Gas1p was found in the mature form in lcb1-100 cells, it was associated tightly with the membrane. In contrast, the immature form of Gas1p in lcb1-100 mutant always showed a certain equilibrium between membrane-associated and -soluble forms. These observations also suggest that the transport is coupled to membrane association. There are two possible models to explain the different behavior of ER and mature Gas1p in lcb1-100 cells. One model is that only the small amount of ER form of Gas1p in lcb1-100 cells that is tightly membrane-associated (for example, by remodeling of GPI-lipid moiety) would be delivered to the Golgi structure and matured. Alternatively, all ER forms of Gas1p could be transported and matured very inefficiently. After arrival in the Golgi compartment, all Gas1p would become tightly membrane-associated because of the different membrane composition. Perhaps sphingolipids are not depleted from the Golgi compartment as severely as from the ER in lcb1-100 mutant cells. So far, we cannot differentiate these possibilities experimentally, but in both cases the weak membrane association could be one of the reasons for the delayed transport.

Ceramides and sphingolipids may have an additional role in GPI-anchored protein transport. GPI-anchored proteins are transported by a different vesicle population than are other
sorptive transport in proteins in the ER could be impaired. This could result in inefficient transport in lcb1-100 cells.

Previously, it was reported that lcb1-100 mutant membranes did not show any defect at the step of ER exit of Gas1p in an in vitro budding assay (3). This in vitro budding assay was carried out at 20 °C using wild-type cytosol. Sphingolipid synthesis is temperature-sensitive in lcb1-100 mutant cells (17), and GPI-anchored protein transport is less defective at lower temperature (3). This could explain the lack of defect seen in the previous study. As shown here, GPI-anchored proteins in lcb1-100 mutant cells at 37 °C behave like peripheral proteins, and a large fraction is released from membranes easily during the assay. Therefore, we could not use the in vitro budding assay to examine ER exit and sorting efficiency in lcb1-100 mutant cells under non-permissive conditions.

There are several possible, not mutually exclusive, ways that ceramide could be required for stable association of GPI-anchored proteins with the ER membrane. Ceramide may be required as a membrane component for the stable association of the GPI-anchor with the membrane. In yeast, both ceramide and GPI-lipid moieties contain C26 fatty acids (32, 33). The hydrophobic interaction between ceramide and the GPI-lipid moiety may be critical for stable membrane association. Another possibility is that ceramide may participate in the formation of lipid raft structures in the ER. Ceramide has been shown to promote membrane microdomain formation in vitro (34). Our results could be interpreted in favor of the hypothesis that microdomain structure is important for GPI-anchored proteins to associate stably with membranes. However, transport of Gas1p from ER to Golgi was not affected significantly in ergosterol biosynthesis mutant cells (21). Our results could be interpreted in favor of the hypothesis that ceramide (32, 33) may be critical for stable membrane association. An-chor remodeling. The significance of remodeling is not yet known, but both remodeling reactions introduce long chain fatty acids onto GPI-anchored proteins (33). This may be nec-

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