Yeast Cells Lacking All Known Ceramide Synthases Continue to Make Complex Sphingolipids and to Incorporate Ceramides into Glycosylphosphatidylinositol (GPI) Anchors*

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In yeast, the inositolphosphorylceramides mostly contain C26:0 fatty acids. Inositolphosphorylerceramides were considered to be important for viability because the inositolphosphorylceramide synthase AURI is essential. However, lcb1Δ cells, unable to make sphingoid bases and inositolphosphorylerceramides, are viable if they harbor SLC1-1, a gain of function mutation in the 1-acetylglieryl-3-phosphate acyltransferase SLC1. SLC1-1 allows the incorporation of C26:0 fatty acids into phosphatidylinositol (PI), thus generating PI Δ, an abnormal, C26-containing PI, presumably acting as surrogate for inositolphosphorylerceramide. Here we show that the lethality of the simultaneous deletion of the known ceramide synthases LAG1/LAC1/LIP1 and YPC1/YDC1 can be rescued by the expression of SLC1-1 or the overexpression of AURI. Moreover, lag1Δ lac1Δ ypc1Δ ydc1Δ (4Δ) quadruple mutants have been reported to be viable in certain genetic backgrounds but to still make some abnormal uncharacterized inositol-containing sphingolipids. Indeed, we find that 4Δ quadruple mutants make substantial amounts of unphysiological inositolphosphorylerphytosphingosines but that they also still make small amounts of normal inositolphosphorylerceramides. Moreover, 4Δ strains incorporate exogenously added sphingoid bases into inositolphosphorylerceramides, indicating that these cells still possess an unknown pathway allowing the synthesis of ceramides. 4Δ cells also still add quite normal amounts of ceramides to glycosylphosphatidylinositol anchors. Synthesis of inositolphosphorylerceramides and inositolphosphorylerphytosphingosines is operated by Aur1p and is essential for growth of all 4Δ cells unless they contain SLC1-1. PI Δ, however, is made without the help of Aur1p. Furthermore, mannosylation of PI Δ is required for the survival of sphingolipid-deficient strains, which depend on SLC1-1. In contrast to lcb1Δ SLC1-1, 4Δ SLC1-1 cells grow at 37 °C but remain thermosensitive at 44 °C.

The inositolphosphorylerceramides (IPCs), mannosyl-IPCs (MIPCs), and inositolphosphoryl-MIPCs (M(IP)2Cs) are major components of the lipidome of the yeast Saccharomyces cerevisiae (1). Moreover, many biosynthetic sphingolipid intermediates have been proposed to function in signal transduction (2–6). Sphingolipids are made by the pathways depicted in Fig. 1, A and B, whereby ceramides are also incorporated into GPI protein anchors (see Fig. 1C).

The acyl-CoA dependent biosynthesis of ceramide is operated by Lag1p and Lac1p (see Fig. 1A), two highly homologous and functionally redundant endoplasmic reticulum proteins that are only active when forming a complex with Lip1p (7–9). Concomitant deletion of LAG1 and LAC1 causes a significant growth defect in the W303 genetic background, and the same double deletion is lethal in the YPK9 background (10, 11). Ypc1p and Ydc1p are two highly homologous ceramides also residing in the endoplasmic reticulum (see Fig. 1A). When the acyl-CoA-dependent ceramide synthases are inhibited or deleted, Ypc1p and Ydc1p can catalyze the reverse reaction, i.e. the condensation of free fatty acids with accumulating long chain bases (LCBs) (12, 13).

The question of whether sphingolipids are required for yeast cell survival has received slightly conflicting answers over the last two decades. A series of seminal studies showed that lcb1Δ cells (see Fig. 1) are auxotrophic for PHS but that a suppressor allele in the lysophosphatidate acyltransferase SLC1 can make them independent of exogenous PHS (14). The suppressor allele SLC1-1 allows lcb1Δ cells to synthesize phosphatidylinositol (PI) with a C26:0 fatty acid instead of C18:1 at the sn-2 position of the glycerol moiety. This lipid, herein dubbed PI Δ, can be mannosylated in the same way as IPCs, unlike normal PI (15). Nevertheless, many sphingolipid-dependent processes such as endocytosis of Lucifer yellow or raft integration, surface transport, and surface stabilization at 37 °C of the essential plasma membrane H+ ATPase Pma1p are fully operational in lcb1Δ SLC1-1 cells (16, 17). These latter studies led to the concept that all essential functions of

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3 The abbreviations used are: IPC, inositolphosphorylerceramide; MIPC, mannosylated IPC; M(IP)2C, inositolphosphoryl-MIPC; AbA, Aureobasidin A; DAG, diacylglycerol; DH5, dihydrosphingosine; FOA, 5 '-fluoroorotic acid; GPI, glycosylphosphatidylinositol; LC8, long chain base; PHS, phytosphingosine; PI, phosphatidylinositol; PI Δ, PI with C26:0 in sn-1; PI Δ, PI with C26:0 in sn-2; aa, amino acids; LM, Lester medium; ESI, electrospray mass ionization; lyso-IPC, inositol-phospho-PHS.
sphingolipids including the stabilization of Pma1p depend on C26-containing lipids but not necessarily on sphingolipids. Although various studies demonstrated that LCB phosphates, M(IP)2Cs, and MIPCs are dispensable (see Fig. 1, A and B) (18–22), IPCs were suspected to be essential because the IPC synthase AUR1 was found to be essential (see Fig. 1B) and because Aureobasidin A (AbA), a highly specific inhibitor of Aur1p, rapidly killed growing yeast cells (23, 24). However, the essentiality of IPCs was questioned when it became clear that the concomitant deletion of all ceramide synthases (LAG1, LAC1, YPC1, YDC1) was not lethal in the W303 background and that W303 lag1Δ lac1Δ ypc1Δ ydc1Δ (W303.4Δ) cells could grow in the presence of AbA (25). It was, however, suspected that these cells grew because they made some abnormal, uncharacterized lipids, which were resistant to mild alkaline hydrolysis and could be labeled metabolically with [3H]inositol, [3H]DHDS, and 32PO4 (25, 26). A more recent study has demonstrated, however, that W303.4Δ cells stop growing on high concentrations of AbA or on AbA at 37 °C and also stop making these abnormal inositolphosphorylsphingolipids (13). These data suggested that the essential functions of IPCs could be taken over not only by PI but also by these abnormal, uncharacterized inositolphosphorylsphingolipids.

In the present study, the abnormal inositolphosphorylsphingolipids are characterized and shown to be dispensable in 4Δ cells only in the presence of SLC1-1. The study documents that through an unknown pathway, W303.4Δ cells, lacking all known ceramide synthases, still make ceramides, which they add to GPI anchors and use for the synthesis of small amounts of normal IPCs.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—S. cerevisiae strains are listed in supplemental Table S1, and plasmids are listed in supplemental Table SII. Cells were grown on rich medium (YPD, YPG, or YPR, containing 2% glucose (D), raffinose (R), or galactose (G) as a carbon source) or synthetic medium (YPD, YPG, or YPR, containing 2% glucose (D), raffinose (R), or galactose (G) as a carbon source) or synthetic medium (SD, SR, or SG (27) or nitrogen base (YNB, Nase (R), or galactose (G) as a carbon source) or synthetic.

RESULTS

SLC1-1 Significantly Increases the Fitness of lag1Δ lac1Δ ypc1Δ ydc1Δ Mutants—The lac1Δ lag1Δ mutant is dead in YPK9 but viable in the W303 background (10, 11). In both backgrounds, we had previously generated quadruple lac1Δ lag1Δ ypc1Δ ydc1Δ mutants containing pBM150-LAG1, an IRA3 vector harboring LAG1, here named W4Δ.LAG1 and Y4Δ.LAG1 (29). It was not possible to force out the LAG1-bearing plasmid from 4Δ.LAG1 in the presence of FOA on...
ordinary complete synthetic media even in the presence of 10% glycerol or the water-soluble synthetic ceramide C6-DHS (not shown; supplemental Fig. S1). However, W4Δ.LAG1 could spontaneously lose the LAG1-bearing plasmid on LM, a synthetic, pH-buffered medium containing 4% glucose and 50 mg/liter inositol (13). As SLC1-1, a gain of function allele of SLC1, can rescue lcb1Δ cells (see the Introduction and Fig. 1), we tested whether it also would improve the fitness of 4Δ cells. This indeed was the case; 4Δ cells harboring SLC1-1 were able to lose pBM150-LAG1 and grow on ordinary complete synthetic media, whereas cells harboring the wild type (WT) allele of SLC1 could not grow on LM, and W4.1.LAC1 was grown in LM containing raffinose as carbon source. 20 A00 units of exponentially growing cells were preincubated for 10 min and labeled with 80 μCi of [3H]inositol for 120 min at 30 °C in SDAuA or SDAuA in the presence of the indicated inhibitors. Proteins were delipidated, and GPI anchor peptides were prepared and treated with nitrous acid (lanes 1–8) or control-incubated (lane 9). The following are indicated below each lane: the radioactivity in lipid extracts containing free lipids (μCi P); the radioactivity in lipids released from GPI anchor peptides (cpm × 10⁻³ A), as well as the radioactivity in ceramide-containing anchors appearing as IPC-3 and IPC-4 (having three or four hydroxyls in their ceramide moiety), expressed as a percentage of total radioactivity per lane (% IPC). The total anchor lipids were spotted onto the TLC and each lane is 10 cpm/lane. The following are indicated below each lane: the radioactivity in lipids containing total radioactivity per lane (% IPC). The total anchor lipids were spotted onto the TLC with less than 10 cpm had been obtained; otherwise 10³ cpm/lane were spotted. Lane 1 contains an aliquot of the lipid extract of SJ21R cells showing that its main free IPS is IPC-4, whereas GPI anchors also contain a large proportion of IPC-3. The TLC was developed in solvent 55:45:5, and spots were visualized by fluorography. MYR, myriocin.

**FIGURE 2.** Temperature sensitivity of 4Δ.SLC1-1 cells. A, cells were plated onto SDAuA plus FOA and incubated at 30 °C for 6 days. B, 10-fold dilutions of cells were plated on LM plates. Plates were incubated at 24, 30, or 37 °C for 4 days and photographed.

**FIGURE 3.** W4Δ.SLC1-1 and Y4Δ.SLC1-1 cells have normal ceramides in their GPI anchors. W4Δ.SLC1-1, lcb1Δ.SLC1-1, and its corresponding parent WT line SJ21R were preincubated in LM, and W4Δ.LAC1 was grown in LM containing raffinose as carbon source. 20 A00 units of exponentially growing cells were preincubated for 10 min and labeled with 80 μCi of [3H]inositol for 120 min at 30 °C in SDAuA or SDAuA in the presence of the indicated inhibitors. Proteins were delipidated, and GPI anchor peptides were prepared and treated with nitrous acid (lanes 1–8) or control-incubated (lane 9). The following are indicated below each lane: the radioactivity in lipid extracts containing free lipids (μCi P); the radioactivity in lipids released from GPI anchor peptides (cpm × 10⁻³ A), as well as the radioactivity in ceramide-containing anchors appearing as IPC-3 and IPC-4 (having three or four hydroxyls in their ceramide moiety), expressed as a percentage of total radioactivity per lane (% IPC). The total anchor lipids were spotted onto the TLC when less than 10 cpm had been obtained; otherwise 10³ cpm/lane were spotted. Lane 1 contains an aliquot of the lipid extract of SJ21R cells showing that its main free IPS is IPC-4, whereas GPI anchors also contain a large proportion of IPC-3. The TLC was developed in solvent 55:45:5, and spots were visualized by fluorography. MYR, myriocin.
W4Δ.SLC1-1 cells incorporate significant amounts of [3H]inositol into GPI anchors, amounts that are comparable with the ones found in W4Δ.LAC1 or S21JR WT cells (Fig 3, lanes 2, 6, and 8) or in BY4742-derived mutants plc1Δ or ppc1Δ, which were found to not have any GPI remodeling defect (supplemental Fig. S4C, lanes 5 – 7). As may be expected, lcb1Δ SLC1-1 cells, although incorporating [3H]inositol into lipids less efficiently than the corresponding S21JR WT cells, made only pG1 type anchors (Fig 1C), i.e. anchors with a C26:0-containing DAG but no anchors containing IPC-3 or IPC-4 (Fig. 3, lanes 7 and 8). In W4Δ.SLC1-1 cells, the incorporation of [3H]inositol into free lipids and GPI anchors could be reduced by myriocin (Fig. 1A), an inhibitor of the serine palmitoyl transferase, and to a lesser extent by AbA (Fig. 1B), but the ratio of ((IPC-3 + IPC-4) total anchor lipids) did not change (% IPC) (Fig. 3, lanes 2 – 5). These two findings can be explained in the sense that (a) in WT cells, only a very small percentage of LCBS is utilized for GPI anchors, and such small amounts are made even in the presence of myriocin and (b) Aur1p is not involved in the addition of ceramides to GPI anchors (36). Only normal ceramide anchors were also found in a fraction of more polar anchor lipids and in other types of 4Δ cell lines (supplemental Fig. S4).

4Δ.SLC1-1 Cells Still Contain Low Amounts of Inositol-containing Sphingolipids—As reported before, W303 lag1Δ lac1Δ and W303.4Δ cells, although devoid of ceramide synthases, make abnormal [3H]inositol-containing lipids, which are resistant to mild alkaline hydrolysis (13, 25, 26). When analyzing lipid extracts of [3H]inositol-labeled cells by TLC/fluorography, we found that similar mild base-resistant lipids are also made in independently generated W4Δ.mSLC1-1, Y4Δ.SLC1-1, and W4Δ.SLC1-1 cells (Fig. 4, A and B; quantitated in supplemental Table S5). Although minor amounts of IPCs and MIPC were detectable (Fig. 4, crosses), 4Δ cells also made a major abnormal mild base-resistant species, lipid a (Fig. 4, asterisk). The synthesis of all base-resistant lipids was partially repressed by myriocin and strongly diminished by AbA (Fig. 4B; supplemental Fig. S5, lanes 1 – 5; data not shown). This suggested that all these lipids including lipid a are sphingolipids and that their biosynthesis requires LCB1 and AUR1 (Fig. 1).

For better characterization, lipid extracts from W303.4Δ and W4Δ.SLC1-1 cells were analyzed by LC-ESI-MS. Searching for ions potentially corresponding to lipid a (Fig. 4, asterisk), we found that W303.4Δ cells contained good amounts of ions corresponding to the expected mass of inositol-phospho-PHS18 (m/z = 558.6) and inositol-phospho-PHS20 (m/z = 586.6) (Fig. 5A). Their fragmentation gave characteristic ions of m/z = 240.9 and 223.0, corresponding to (inositolphosphate-H2O) and (inositolphosphate-2 H2O), whereas fragments 396.3 and 378.3 are the product of neutral losses of 180.3 and 162.3, which correspond to the loss of inositol and (inositol-H2O), respectively (Fig. 5B). Substantial amounts of the same compound were also found in the other 4Δ strains tested (supplemental Fig. S6A). Inositol-phospho-PHS was recently found in the fatty acid elongation mutants elo2Δ and elo3Δ and was named lyso-IPC (39). The elo3Δ mutant has difficulty making ceramides because C26-CoA and C24-CoA,
the optimal substrates for Lac1p/Lag1p, are lacking (40)). As shown in supplemental Fig. S6B, a direct comparison showed that the relative percentage of lyso-IPC among the total of negative ions was 35-fold higher in W303.4Δ cells than WT cells and 5-fold higher than in elo3Δ cells. Lyso-IPCs are expected to be mild base-resistant and to migrate in TLC significantly less than IPCs, as is the case for lipid a (Fig. 4). Lyso-IPCs therefore are likely corresponding to the major mild base-resistant [3H]inositol-, [3H]DHS- and [32P]O4- labeled abnormal sphingolipids of Δ cells previously described as lipids a and b and lipids X1 and X2 in W303 lag1Δ lac1Δ and W303 Δ cells (13, 25, 26). Lipid b was only occasionally observed in [3H]inositol-labeled extracts of Δ cells (e.g. Fig. 4B, lane 10, double asterisks). Lipid profiling using mass spectrometry also showed that W303.4Δ cells have very high levels of LCBs and LCB phosphates with regard to elo3Δ and WT cells and that they are low in phosphatidylserine (supplemental Fig. S6B).

The LC-ESI-MS analysis also showed that Δ cells still contain small amounts of IPC44 and IPC46 with three, four, or five hydroxyl groups in their ceramide moiety, the ratio of intensities of IPC/PI in Δ mutants being 1.3–1.4% of the ratio observed in WT cells (Fig. 6, A and B). Fragmentation of IPCs in Δ cells resulted in characteristic neutral losses of 162 Da (inositol–H2O) and 180 Da (inositol), confirming their identity as IPCs (not shown). The drastic reduction in the IPC/PI ratio is the result of a 40-fold reduction in IPC intensities and a concomitant ~2-fold increase in PI intensities. This can be explained by the fact that in WT cells, 40% of PI is used to make complex sphingolipids (Fig. 1) (41). IPC-3 is usually very minor in WT cells but was accounting for more than a third of IPCs in Δ cells (Fig. 6B). Moreover, Δ cells contained 15.5 times more PIs with a total of 42 and 44 carbon atoms in the two fatty acids than WT (Fig. 6C). This is expected because only few very long chain fatty acids can be utilized for making sphingolipids in Δ cells, and they thus spill over into PI. Interestingly, the PI44 species were of similar abundance whether or not SLC1-1 was present, indicating that the increased fitness of Δ-bearing cells is not due to higher amounts of C26-containing PI but rather to the presence of some PI′ (having C26:0 in sn-2) together with PI (i.e. PI with C26:0 in sn-1), the latter being the only species present in cells not bearing SLC1-1.

**Figure 6.** W4 Δ, SLC1-1 and W303.4Δ cells contain small amounts of normal IPCs. Indicated strains were grown in LM at 30 °C. Lipids were extracted (41), and aliquots corresponding to 0.1 A530 units of cells from each strain were analyzed by LC-ESI-MS on a Thermo Scientific LTQ XL mass spectrometer. Intensities used to calculate these ratios are in supplemental Table SIV. B, the relative abundance of different species of IPCs was expressed as a percentage of the sum of all IPCs in each strain. C, the relative abundance of different species of PIs was expressed as a percentage of the sum of all PIs in each strain. Note that PIs elute in the same fractions as IPCs from LC.
the synthetic complete LM. Note that IPCs could be taken up, hydrolyzed by Isc1p, and then utilized by Aur1p during a metabolic frame. The finding that we could not clone W303.4Δ cells, hydrolyzed by Isc1p, and then utilized by Aur1p during a metabolic frame with either yeast lipid extract or boiled yeast microsomes as a support. No generation of ceramides was observed (not shown).

We found that we could not clone W303.4Δ cells by micro-manipulation, possibly because they die upon physical separation of daughter from mother cells. We therefore wanted to be sure that the strains we were looking at were not contaminated by some other yeast species. Low stringency PCR followed by DNA sequencing showed, however, that all DNA sequences we obtained are present in the genome of S. cerevisiae, making it highly unlikely that another species is contaminating our 4Δ strains (supplemental Fig. S8).

Overexpression of AUR1 Can Rescue Viability of YPK9.4Δ Cells—The presence of high amounts of lyso-IPCs in 4Δ cells raised the possibility that the ability to make high amounts of these lipids may decide whether a given genetic background can tolerate the deletion of all ceramide synthases, as is the case for W303 but not for YPK9. DNA sequencing of the open reading frame of the genomic AUR1 in W303.4Δ (FBY958-Lnew) as well as in YΔLAG1, a YPK9 lag1Δ lac1Δ strain unable to loose the covering pBM150-LAC1 plasmid, showed that both strains had the identical AUR1 coding sequence. This did not exclude that W303 contained higher amounts of the IPC synthase Aur1p or of Kei1p, the second essential subunit of the IPC synthase (43). As overexpression of AUR1 can suppress the growth defect of kei1-1 mutants, we tried to see whether the introduction of extra copies of AUR1 into Y4ΔLAG1 cells would allow them to lose the pBM150-LAG1 plasmid on FOA. This indeed was the case. The thus generated Y4ΔAUR1 strain also made significant amounts of lyso-IPCs (supplemental Fig. S6A). Assuming that the affinity of Aur1p for free LCBs is much lower than that for ceramides, one would predict that lyso-IPCs are made efficiently only by cells having high concentrations of LCBs and low levels of ceramides, conditions that are met in all 4Δ cells. The fact that Y4ΔAUR1 cells are viable, whereas YPK9.4Δ cells are inviable, also raises the possibility that lyso-IPCs, like PI", can act as a substitute for normal IPCs, but we cannot exclude that the overexpression of Aur1p allows for more efficient synthesis of essential IPCs rather than lyso-IPCs.

4ΔSLC1-1 Cells Are Resistant to High Concentrations of Aureobasidin A—Various single-point mutations in AUR1 were reported to make WT cells resistant to very high concentrations (>20 or 25 μg/ml) of AbA, i.e. 100–500-fold more resistant than WT cells, and this without affecting their sensitivity to other drugs (44, 45). This argues that AbA is a specific inhibitor of IPC synthase. AbA suppresses the biosynthesis of lyso-IPCs in W303.4Δ (13) as well as W4ΔSLC1-1 cells (Fig. 4B) and blocks growth of W303.4Δ cells (13). As shown in Fig. 8A, 4ΔSLC1-1 cells are partially resistant to AbA, but AbA clearly reduces their cloning efficiency. However, W4ΔSLC1-1 cells could be further propagated on high concentrations of AbA (Fig. 8B), much in contrast to W303.4Δ cells lacking SLC1-1 (13). These data suggest that
lyso-IPCs or else the small amounts of IPCs/MIPCs contribute to the robustness of W4Δ.SLC1-1 cells but that the introduction of SLC1-1 has rendered these sphingolipids dispensable for cell survival. ∆ cells were less sensitive to myriocin than to AbA (Fig. 8A), in keeping with the finding that myriocin could not suppress the addition of ceramides to GPI anchors (Fig. 3, lane 3) nor the synthesis of small amounts of MIPC (Fig. 8). Myriocin could not suppress the addition of ceramides to GPI anchors (Fig. 3, lane 3) nor the synthesis of small amounts of MIPC (Fig. 8).

**FIGURE 8. Growth of ∆.SLC1-1 cells is compromised by Aureobasidin A but not abolished.** A, The indicated strains were precultured at 30 °C in LM. Starting with a cell suspension with an A600 of 10, serial 10-fold dilutions were plated on LM plates containing AbA, myriocin, or methanol (MeOH). Plates were incubated at 30 °C and photographed after 3 and 6 days. ∆ cells from the colonies growing on AbA (next to the white asterisk in A) were streaked out onto new plates containing or not 2.5 µg/ml AbA, together with W303 WT cells. Plates were incubated at 24 °C and photographed after 3 days (left and middle); growing W303 ∆ cells were again taken from the middle plate, streaked onto a fresh plate, and once more cultured for 3 days (right).

**MANNOSYLATION OF C26:0 CONTAINING PHOSPHATIDYLINOSITOL**

The simplest hypothesis to explain this finding is that SLC1-1p makes some phosphatidic acid with C26:0 in sn-2 (PA2), that this PA2 is transformed into CDP-DAG, and that only PI synthase (PIS1) is able to utilize CDP-DAG but phosphatidylserine synthase (CHS1) is not. Based on the partial AbA sensitivity of ∆.SLC1-1 cells, we considered the alternative hypothesis that the PA2 would be degraded by Pah1p to DAG containing C26:0 in sn-2 and that in the absence of ceramides, DAG may be mistaken by IPC synthase Aur1p as a ceramide so that Aur1p would transfer inositolphosphate from a PI onto this DAG, thereby generating PI*. To test for this, we exploited the fact that the presence of MPI* is evidence for the synthesis of PI*. Indeed, although PI* (i.e. a PI with C26:0 in sn-1) does not serve as an acceptor for mannoses transferred by Csg1p or Chs1p, PI* (with C26:0 in sn-2) does (15, 46). Thus, we decided to test whether MPI* synthesis in lcb1Δ cells can be blocked by AbA. Metabolic labeling of yeast with [3H]mannose is feasible only in pmi40 cells, unable to make mannose at 37 °C. Labeling of pmi40p cells generates one major and two minor radioactive species of [3H]MIPC as well as [3H]M(IP)2C (Fig. 9A, lane 2, and 9C, lane 16). As expected, adding myriocin or AbA to the labeling reaction greatly diminishes the labeling of these lipids (Fig. 9A, lanes 2, 4, and 6). (The origin of the band migrating as the upper of the two MIPC bands after mild base hydrolysis is not known (Fig. 9A, lanes 3, 5, and 7).) In pmi40 lcb1Δ cells, no labeling of MIPC or M(IP)2C is observed, but one can see three mild base-sensitive bands migrating at and slightly above the position of MIPC; these bands most likely correspond to lyso-MPI, lyso-MIP(C), and lyso-MIP(C)2 (Fig. 9D, lanes 23 and 25). Thus, Aur1p is not required to make mannosylated PI*, and hence to make PI*, and this suggests that the exclusive incorporation of C26 into PI seen in SLC1-1 cells reflects the fact that Pis1p can use CDP-DAG with a C26 sn-2, whereas Chs1p cannot. Alternatively, C26 may be introduced into PI through a SLC1-1-dependent lipid deacylation-reacylation cycle as Sli1p was shown to acylate lyso-PI and to prefer this substrate to lyso-phosphatidylcholine or lyso-phosphatidylethanolamine (47). At any event, the partial AbA sensitivity of Y4Δ.SLC1-1 and W4Δ.SLC1-1 (Fig. 8A) cannot be explained by an effect of AbA on the biosynthesis of PI*.

**MANNOSYLATION OF C26:0 CONTAINING PHOSPHATIDYLINOSITOL HELPS THE SURVIVAL OF SPHINGOLIPID-DEFICIENT CELLS**—Individual deletions of either CSG1 or CHS1, the functionally redundant IPC-specific mannosyltransferases, have little effect on MIPC biosynthesis, whereas simultaneous deletion of both genes totally abolishes mannosylation of IPC (Fig. 1B). Also, csg1Δ chs1Δ cells grow normally, showing that mannosylation of glycerophospholipids (14).
and (FBY952) cells were growing exponentially in LM supplemented with 2 mM PI (20, 21). We assumed that PI IPCs and formation of MIPC are not required for cell survival. Myriocin (Myr, 40 μg/ml) or AbA (1 μg/ml) were added, and cells were further grown for 100 min. 10 A600 units of cells were washed and resuspended in LM with 2% pyruvate, glucose reduced to 0.2%, 20 μg/ml tunicamycin, and inhibitors. Cells were further preincubated for 20 min at 37 °C. Then, [2-3H]mannose (100 μCi) was added, and cells were labeled during 60 min at 37 °C. Lipids were extracted, deacylated with NaOH (+) or control-incubated (!), and analyzed by TLC/fluorography. C, indicated strains were labeled with [2-3H]inositol (I) or [2-3H]mannose (M). D, the preculture additionally contained 3 μM PHS; AbA was present during preculture and labeling (lanes 22 and 23) or only during labeling (lanes 24 and 25). MIPCs are marked with an asterisk, and the regions containing mannosyl-Ph (MPI) are boxed.

IPC and formation of MIPC are not required for cell survival (20, 21). We assumed that PI* is mannosylated by Csg1 or Csh1p. To see whether the MPI* forms generated by SLC1-1 cells lacking sphingolipids are important for cell survival, we treated csh1Δ, csg1Δ, csh1Δ csg1Δ, and WT cells expressing SLC1 or SLC1-1 with myriocin. As shown in Fig. 10, all mutants not expressing the suppressor allele SLC1-1 were highly sensitive to myriocin. SLC1-1 was able to rescue cells expressing Csg1, whereas csg1Δ SLC1-1 and csg1Δ csh1Δ SLC1-1 cells were completely unable to grow. This result suggests that mannosylation of PI*, i.e., the biosynthesis of MPI*, is essential for the viability of cells lacking sphingolipids, and the presence of the minor mannosyltransferase Csh1p is not sufficient for cell survival on myriocin, either because it is catalytically less active than Csg1p or because it does not utilize PI* as a substrate. Lipid extract of Δ4 cells contained negative ions of m/z 1137, which corresponds to the expected m/z of manno-syl-PI44:1 with oleic acid in sn-1 and C26:0 in sn-2, albeit in quantities too low to allow fragmentation (not shown).

W4Δ SLC1-1 Cells Are Heat Shock-intolerant—Free LCBs, LCB phosphates, as well as ceramides get elevated when yeast cells are heat-shocked by a shift from 24 to 37 °C (18, 48, 49). As summarized in Table 1, lcb1-100 mutant cells cannot grow at 37 °C and other stress conditions (50). Similarly, lcb1-100 mutant cells show no increase in the levels of PHS and DHS during heat stress, cannot grow at 37 °C, and show reduced survival during stronger heat stresses (51–53). Moreover, lcb1-100 mutant cells cannot initiate translation of Hsp70 mRNAs after a heat-induced global translation arrest (54).

FIGURE 10. SLC1-1 and IPC mannosyltransferases both contribute to the viability of cells when sphingolipid biosynthesis is blocked. Strains FBY999–F8Y9110 were grown to exponential phase, and suspensions of an A600 of 10 were serially diluted by 10-fold dilution steps and plated on LM plates without or with 40 μg/ml myriocin (myr.). The four strains in each panel are the four spores of a tetrapisper tetrad generated by crossing a csg1Δ with a csh1Δ strain harboring the vector indicated on top of each panel. The csg1Δ csh1Δ double mutants are labeled ccΔ. Plates were incubated at 24 °C for 3 and 6 days and photographed. cen, centromeric vector.
SLC1-1-dependent lipid. To explore whether SLC1-1-dependent lipids also confer resistance to a stronger heat stress, we tested the plating efficiency of W4Δ.SLC1-1 cells having been cultured at 24 °C and then having been shifted for various times to 44 °C following the protocol previously used for lcb1-100 cells by Friant et al. (53) (Fig. 11A). W303 WT cells resisted this heat treatment despite being notorious for harboring a truncated SSD1 allele, Ssd1p being a cochaperone for Hsp104p, a mutation that renders W303 more heat-sensitive than other WT strains (55) (Fig. 11). In contrast, W4Δ.SLC1-1 cells were found to be sensitive to a temperature shift to 44 °C. The time required to kill cells varied from 12 to 30 min. Curiously, cells survived better when plated on PHS then without (not shown). Moreover, the overexpression of UBI4, encoding for ubiquitin, could not rescue the heat sensitivity of W4Δ.SLC1-1 cells (Fig. 11B), whereas the same plasmid had been reported to rescue lcb1-100 cells under the same heat stress conditions, presumably by accelerating the ubiquitinylation and proteosomal degradation of aggregated proteins (53). Inspection of the overview in Table 1 shows that only W4Δ.SLC1-1 cells grow at 37 °C. This suggests that for cells lacking ceramide and IPCs, both an SLC1-1-dependent lipid and significant PHs levels need to be present to allow cells to grow at 37 °C. Our data further suggest that different kinds of damage are caused by severe heat stress in W4Δ.SLC1-1 and lcb1-100 cells. W4Δ.SLC1-1 cells having very high levels of LCBS may not have any problem inducing translation of mRNAs for heat shock proteins and therefore are not helped by UBI4 overexpression, but they must be unable to survive 44 °C because of the almost complete lack of ceramides and complex sphingolipids. In contrast, membranes of lcb1-100 SLC1-1 cells, which still have considerable amounts of complex sphingolipids, may better resist the high temperatures but have difficulty reintegrating heat shock proteins because they are unable to raise LCBS during heat stress (52). Altogether the data illustrate that even the concomitant presence of large amounts of PHS, of small amounts of IPCs, of lyso-IPCs, and of SLC1-1-dependent lipids cannot compensate for the absence of the normal sphingolipids during a strong heat stress at 44 °C.

**DISCUSSION**

The present report identifies the most abundant abnormal sphingolipids of lagΔ lac1Δ ypcΔ ydc1Δ cells as lyso-IPCs. Lyso-IPCs indeed are expected to be metabolically labeled with radioactive inositol, DHS, and phosphate, as reported for lipids a and b, X1, and X2 before (25, 26). The synthesis of lyso-IPCs is blocked by AbA (Fig. 4B; supplemental Fig. S5) and lyso-IPCs therefore seem to be made by Aur1p. Aur1p has previously been shown to use not only the physiological C26- and C24-containing ceramides but also ceramides with shorter fatty acids of 2, 6, or 16 carbon atoms (13, 56), and this report clearly shows that in the absence of other substrates, Aur1p can even use free LCBS, albeit with a preference for C20 containing LCBS. However, we could not find ions corresponding to mannosylated lyso-IPCs, suggesting that lyso-IPCs are not a substrate for Csg1p or Csh1p.

This study was triggered by the stunning observation that 4Δ cells continue to add ceramides to GPI anchors (Fig. 4). Comparison of the relative amounts of [3H]inositol in GPI proteins having ceramide anchors (Fig. 3, lanes 2 and 6) and in free IPCs (supplemental Table SV) indicates that the loss of ceramide synthases brings IPCs down 15–25-fold, whereas ceramide-containing GPI anchors are down only 2-fold (see supplemental calculation). It therefore appears that cells lacking known ceramide synthases continue to make small amounts of ceramides and incorporate them preferentially into GPI anchors, whereas they cease by and large to make IPCs. Two alternative hypotheses can explain the preferential...
incorporation of ceramides into GPI anchors. Either the Cwh43p ceramide remodelase has a higher affinity for ceramides than Aur1p, or the ceramides of Δ4 cells are generated by a process, which channels them preferentially into the ceramide remodelase. At any event, the TLC mobility of IPCs present in GPI anchors of Δ4 cells and WT cells is the same, suggesting that the unknown enzyme generating ceramides in Δ4 cells makes ceramides containing very long chain fatty acids, as are found in WT GPI anchors (57). It may be that Cwh43p, the ceramide remodelase, has a high specificity for C42- and C44-ceramides because it accepts neither ceramides nor acyl-ceramides. As this is not the case, we can assume that the unknown enzyme generating ceramides in Δ4 cells would make ceramides with shorter fatty acids, they should be transformed into IPCs. Either the unknown ceramide synthase of Δ4 cells makes ceramides with shorter fatty acids, they should be transformed into IPCs. As this is not the case, we can assume that the unknown enzyme generating ceramides in Δ4 cells is specific for very long chain fatty acids.

Aureobasidin A at 2.5 μg/ml stops the growth of W303.Δ4 cells (13), whereas it slows, but does not stop, the growth of W4.AΔSLC1-1 cells (Fig. 8B). The growth inhibitory effect of AbA cannot be due to an influence on GPI anchoring as it was shown that AbA does not block ceramide incorporation into GPI anchors even at concentrations up to 10 μg/ml (36). It is also becoming clear from our studies that Aur1p is not required for making PI’ (Fig. 9), and so the inhibitory effect of AbA on the growth of Δ4ΔSLC1-1 cells must be achieved through blocking the synthesis of IPCs and/or lyso-IPC, not of PI’ and MPI’. Although the immediate toxic effect of AbA in WT cells is most likely due to the toxicity of accumulating ceramides (13, 25), this kind of toxic effect of AbA can safely be excluded in Δ4 cells. The data therefore suggest that lyso-IPC and/or IPCs make an essential contribution to the cell viability of W4.AΔSLC1-1 cells, a contribution that becomes dispensable only if cells harbor SLC1-1 (Fig. 8B).

PI’ only represents 1–2% of PI in SLC1-1 lcb1Δ cells (15). Although it seemed reasonable to assume that lcb1ΔΔSLC1-1 cells need PI’, MPI’, and/or inositol-phospho-MPI’ for cell survival, it was not formally excluded that they only require PA’ or DAG’ or just a pathway that reduces toxic levels of C26:0 (15). However, the fact that mannosylation of PI’ is important for survival of SLC1-1 cells lacking LCBs (Fig. 10) strongly suggests that MPI’ and/or inositol-phospho-MPI’ are the life-saving lipids in cells lacking complex sphingolipids. In WT cells, about 1% of PI carries a C26 fatty acid in sn-1 (46), and the percentage of this PI’ is massively increased in lacΔΔlag1ΔΔ cells (26, 29) (Fig. 6C). However, it seems that PI’ is not able to rescue lcb1Δ or YPK9.Δ4 cells, but only PI’, carrying a C26:0 in sn-2. It may be speculated that the PI’ fails to rescue lcb1Δ or YPK9.Δ4 cells not because its biophysical properties are very different from PI’ but because it is not a substrate for Csg1p and therefore cannot be mannosylated to yield the essential mannosylated forms of PI. Although SLC1-1 expression had the same life-saving effect on YPK9.Δ4 cells as on lcb1Δ cells, we could not directly confirm that mannosylation of PI’ is also crucial for the survival of Δ4 cells because Δ4 cells are not very robust and the introduction of additional mutations is difficult. Further efforts will be needed to identify the enzyme producing the ceramides in Δ4 cells.

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