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. Inositolphosphorylceramides were considered to be important for viability because the inositolphosphorylceramide synthase AUIRl is essential. However, lcb1Δ cells, unable to make sphingoid bases and inositolphosphorylceramides, are viable if they harbor SLC1-1, a gain of function mutation in the 1-acylglycerol-3-phosphate acyltransferase SLC1. SLC1-1 allows the incorporation of C26:0 fatty acids into phosphatidylinositol (PI), thus generating PIt, an abnormal, C26-containing PI, presumably acting as surrogate for inositolphosphorylceramide. Here we show that the lethality of the simultaneous deletion of the known ceramide syntheses LAG1/LAC1/LIP1 and YPC1/YDC1 can be rescued by the expression of SLC1-1 or the overexpression of AUIR1. 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 inositolphosphorylphytosphingosines but that they also still make small amounts of normal inositolphosphorylceramides. Moreover, 4Δ strains incorporate exogenously added sphingoid bases into inositolphosphorylceramides, 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 inositolphosphorylceramides and inositolphosphorylphytosphingosines is operated by Aur1p and is essential for growth of all 4Δ cells unless they contain SLC1-1. PIt, however, is made without the help of Aur1p. Furthermore, mannosylation of PIt 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.

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3 The abbreviations used are: IPC, inositolphosphorylceramide; MIPC, mannosyl-IPC; MIPCs, mannosylated MIPC; M(IP)2C, inositolphosphoryl-MIPC; AbA, Aureobasidin A; DAG, diacylglycerol; DHS, dihydrosphingosine; FOA, 5'-fluoroorotic acid; GPI, glycosylphosphatidylinositol; LCB, long chain base; PHS, phytosphingosine; PI, phosphatidylinositol; PIt, PI with C26:0 in sn-1; PIt, PI with C26:0 in sn-2; aa, amino acids; LM, Lester medium; ESI, electrospray mass ionization; lyso-IPC, inositol-phospho-PHS.
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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 MIPC 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]DHS, and 32P orthophosphate, M(IP)2Cs, and MIPCs are dispensable (see Fig. 1, A and B).

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 SI, and plasmids are listed in supplemental Table SII. Cells were grown on rich medium (YPD, YPG, or YPR, containing 2% glucose), 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 “LM”, a complete synthetic medium buffered at pH 6.0, containing 2 mg/liter of uracil (U), and adenine (A), or high amounts (50 mg/liter) of uracil (U), and adenine (A), or high amounts (50 mg/liter) of inositol (I) instead of only 2 mg/liter. We also used “Lester medium” (LM), a complete synthetic medium buffered at pH 6.0, containing 4% glucose or 4% galactose, 50 mg/liter inositol but without LCbs and Tergitol (15). LM was designed for cells unable to make LCbs. Methionine was often omitted to increase the transcription from the MET25 promoter (28).

Construction of Strains and Plasmids—W4Δ strains were constructed from 4Δ lag1Δ strains described before (29). After transfection of plasmids containing SLC1, SLC1-1, or AUR1, the covering plasmids containing LAG1 and/or LAC1 were shuffled out using 5’-fluoroorotic acid (FOA). Unfortunately, the FOA selection does not work on LM. To construct plasmids containing SLC1 or SLC1-1, the open reading frames were amplified by PCR using oligonucleotides SLC1.F1 and SLC1.R1 (supplemental Table SIII) and genomic DNA from S21R or lcb1Δ SLC1-1 as templates. The PCR products were doubly digested with SpeI and XhoI and ligated into the covering plasmids containing LAG1 or LAC1 and SLC1.R1 (supplemental Table SIII) and genomic DNA from S21R or lcb1Δ SLC1-1 as templates. The PCR products were doubly digested with SpeI and XhoI and ligated into the

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 URA3 vector harboring LAG1, here named W4ΔLAG1 and Y4ΔLAG1 (29). It was not possible to force out the LAG1-bearing plasmid from 4ΔLAG1 in the presence of FOA on
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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 loose 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 loose pBM150-LAG1 and grow on ordinary complete synthetic media, whereas cells harboring the wild type (WT) allele of SLC1 grew only very slowly or not at all (Fig. 2A; supplemental Fig. S2). 4Δ cells harboring a centromeric SLC1-1 vector in the W303 and YPK9 backgrounds were called W4Δ.SLC1-1 and Y4Δ.SLC1-1; the ones containing a SLC1-1 multicopy vector were called W4Δ.mSLC1-1 and Y4Δ.mSLC1-1. Their genotypes were confirmed by PCR (supplemental Fig. S3). Overall, W4Δ.SLC1-1 cells were more robust than Y4Δ.SLC1-1 because they grew to higher densities in liquid media and grew at 37 °C (not shown; Fig. 2B). Thus, even after the introduction of SLC1-1, YPK9 cells tolerate the deletion of ceramide synthases less well than W303 cells.

4Δ.SLC1-1 Cells Still Add Normal Ceramides to the GPI Anchors—GPI anchoring is essential for yeast, survival and most GPI anchors of yeast possess an inositolphosphorylceramide, which is introduced into the anchor by lipid remodeling (32). CWH4Δ3 and other mutants, which do not incorporate ceramide into GPI anchors, are viable (33–35), indicating that ceramide is not an essential component of GPI anchors. The incorporation of ceramides into GPI anchors supposedly is analogous to the IPC synthase reaction carried out by Aur1p, but it seems to be independent of Aur1p because it cannot be inhibited by AbA (36). As 4Δ.SLC1-1 cells should not make ceramides, we wanted to see what lipids they utilize for GPI anchoring. Anchor lipids can be analyzed by mass spectrometry, TLC, and HPLC. Here we present a TLC protocol that allows the resolution of anchor lipids in cell samples.

FIGURE 2. Temperature sensitivity of 4Δ.SLC1-1 cells. A, cells were plated onto SDaaUA plus FOA and incubated at 30 °C for 6 days. 8-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 parental WT line SJ21R were precultured in LM, and W4Δ.LAC1 was grown in LM containing raffinose as carbon source. 20 A600 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 SDaaUA or SRaaUA 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 F), 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 106 cpm had been obtained; otherwise 105 cpm/lane were spotted. Lane 1 contains an aliquot of the lipid extract of SJ21R WT cells showing that its main free IPC 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, myricin.
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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 pgclΔ, 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 lagΔ lacΔ and W303.Δ 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-H$_2$O) and (inositolphosphate-2H$_2$O), 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-H$_2$O), 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, ...
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The optimal substrates for Lac1p/Lag1p, are lacking (40). As shown in supplemental Fig. S6A, a direct comparison showed that the relative percentage of lyso-IPCs among the total of negative ions was 35-fold higher in W303.4Δ cells than WT cells and 5-fold higher than in SLC1-1 cells (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.

4Δ Cells Make Ceramides from Exogenously Added Long Chain Bases—The presence of small amounts of normal IPCs and ceramides in 4Δ cells is puzzling because cells lack all of the known ceramide synthases. We have struggled with the question of whether the cells themselves make these lipids or whether they are taken up from the media. To resolve this, we tried to force 4Δ cells to make IPCs from exogenously added precursors by specifically blocking either fatty acid or LCB biosynthesis. Although cerulinin, an inhibitor of fatty acid synthase, killed the cells in the presence of exogenous fatty acids, cells were quite resistant to myriocin (see below, Fig. 8A). As shown in Fig. 7A, the IPCs made by 4Δ cells in the presence of myriocin and LCBs containing either 18 or 20 carbon atoms were quite different. In the presence of DHS18, the cells mainly made IPCs with 44 carbon atoms and three, four, or five hydroxyls in the ceramide; in the presence of DHS20, they made mainly IPCs with 46 carbon atoms and three hydroxyls in the ceramide. Although the data in Fig. 7 clearly demonstrate that 4Δ cells can make ceramides, they do not exclude that part of the IPCs of 4Δ cells are derived from sphingolipids contaminating cells previously described as elο3Δ cells (Fig. 6A). Fragmentation of IPCs in 4Δ 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 4Δ cells (Fig. 6B). Moreover, 4Δ 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 very few very long chain fatty acids can be utilized for making sphingolipids in 4Δ 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 SLC1-1-bearing strains 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.

The LC-ESI-MS analysis also showed that 4Δ 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 4Δ mutants being 1.3–1.4% of the ratio observed in WT cells (Fig. 6, A and B). Fragmentation of IPCs in 4Δ 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 4Δ cells (Fig. 6B). Moreover, 4Δ 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 very few very long chain fatty acids can be utilized for making sphingolipids in 4Δ 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 SLC1-1-bearing strains 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.

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cells and always below 25. 6774
abolic labeling experiment with [3H]inositol. However, fur-
hydrolyzed by Isc1p, and then utilized by Aur1p during a met-
ported are IPCs (units of cells from each strain were analyzed by LC-ESI-MS as in Fig. 6. Re-
periodically during the first 8 h, in proportion to the cell density, so that the
tracts from WT cells did not improve the growth of 4
growth media with crude, sphingolipid-containing lipid ex-
sumption of essential IPCs rather than lyso-IPCs.

Indicated strains were grown in LM at 30 °C in the presence (−) or absence (−) of indicated reagents (myriocin (Myr), 40 µg/ml) and either
B
HLS18 or DHS20. Cells were seeded at an A500 of 0.1, and DHS was added
typically, we incubated [3H]C16:0 or [3H]C16:0-CoA with
−C18 C20 −C18 C20
W303.4Δ W4Δ.SLC1-1

Perhaps the introduction of extra copies of AUR1 into
Y4Δ.LAG1 cells would allow them to lose the pBM150-LAC1 plasmid. showed
that both strains had the identical AUR1 coding sequence.

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 (FHY958-
Lnew) as well as in Y2Δ.LAC1, a YPK9 lag1Δ lac1Δ strain unable to loose the covering pBM150-LAC1 plasmid, showed
this did not exclude that W303 contained higher amounts of the IPC synthase Aur1p or Ke11p, 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
differ between 2.1 and 4. Lipid extracts corresponding to 0.1 A500
units of cells from each strain were analyzed by LC-ESI-MS as in Fig. 6. Report-
duced IPCs (A) and lyso-IPCs (B). The untreated cells represent the same
data as reported in the legend for Fig. 6. No species with only two hydroxyls
or with double bonds were detected.

the synthetic complete LM. Note that IPCs could be taken up,
hydrolyzed by Isc1p, and then utilized by Aur1p during a met-
abolizing experiment with [3H]inositol. However, fur-
ther arguments against an external source of ceramides in 4Δ cells are the following. 1) lcb1Δ SLC1-1 cells grown in the same medium cells do not make those lipids (supplementary Fig. S7), nor do they incorporate ceramides into GPI anchors (Fig. 3, lane 7). 2) Previous attempts to rescue the growth phenotype of lcb1Δ cells by adding ceramides (DHS-C6, DHS-C24, DHS-C26-OH) or M(IP)2C to the growth medium were unsuccessful (42). 3) Growth media extracted with ethyl ether to remove apolar lipid contaminants supported normal cell growth of 4Δ cells, and the deliberate supplementation of growth media with crude, sphingolipid-containing lipid extracts from WT cells did not improve the growth of 4Δ cells (not shown). 4) In some experiments, myriocin treatment abrogated biosynthesis of all mild base-resistant IPCs in 4Δ cells (supplemental Fig. S5, lanes 1–4). In summary, all our

findings argue in favor of an unknown further pathway in 4Δ cells allowing them to synthesize small amounts of ceramides.

To see whether ceramides could be generated non-enzymatically, we incubated [3H]C16:0 or [3H]C16:0-CoA with LCBs during 4 h at physiological pH 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 separa-
tion of daughter from mother cells. We therefore wanted to be sure that the strains we were looking at were not contamin-
ated by some other yeast species. Low stringency PCR fol-
lowed by DNA sequencing showed, however, that all DNA sequences we obtained are present in the genome of S. cerevi-
siae, making it highly unlikely that another species is contami-
nating our 4Δ strains (supplemental Fig. S8).

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whether the introduction of extra copies of AUR1 into
Y4Δ.LAG1 cells would allow them to lose the pBM150-
LAG1 plasmid on FOA. This indeed was the case. The thus
-generated Y4Δ.AUR1 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 Y4Δ.AUR1 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.

4Δ.SLC1-1 Cells Are Resistant to High Concentrations of Auroebasidin 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 W4Δ.SLC1-1 cells (Fig. 4B) and blocks growth of W303.4Δ cells (13). As shown in Fig. 8A, 4Δ.SLC1-1 cells are partially resistant to AbA, but AbA clearly reduces their cloning efficiency. However, W4Δ.SLC1-1 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
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Glycerophospholipids (14). The simplest hypothesis to explain this finding is that SLC1-1p makes some phosphatidic acid with C26:0 in sn-2 (PA”), that this PA” is transformed into CDP-DAG”, and that only PI synthase (PIS1) is able to utilize CDP-DAG” but phosphatidylserine synthase (CHO1) is not. Based on the partial AbA sensitivity of ΔΔSLC1-1 cells, we considered the alternative hypothesis that the PA” 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 inositolphosphatase 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 SLC1-1 cells can be blocked by AbA. Metabolic labeling of yeast with [3H]mannose is feasible only in pmi40Δ-cells, unable to make mannose at 37 °C. Labeling of pmi40Δ-cells generates one major and two minor radioactive species of [3H]MIPC as well as [3H][MIP]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 SLC1-1 cells, no labeling of MIPC or (MIP)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 MPI” (Fig. 9B, boxed, lanes 12 and 13, and 9C, lane 17). This MPI” is sensitive to phospholipase A2 (not shown) and is labeled equally strongly also in the presence of myriocin or AbA (Fig. 9B, lanes 8, 10, and 12). Also, several other mild base-sensitive and one mild base-resistant band are present in pmi40 lcb1Δ SLC1-1 but not pmi40 cells; some may correspond to lyso-MPI”s, pmi40 lcb1Δ SLC1-1 precultured and/or labeled in the presence of PHS generated both MIPCs and MPI” (Fig. 9D). In this case also, AbA blocked the biosynthesis of the major species of MIPC but not of MPI” (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 in sn-2, whereas Cha1p cannot. Alternatively, C26 may be introduced into PI through a SLC1-1-dependent lipid deacylation-reacylation cycle as Scl1p was shown to acylate lyso-PI and to prefer this substrate to lysophosphatidylcholine or lysophosphatidylethanolamine (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 CSH1, 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, csh1Δ csg1Δ cells grow normally, showing that mannosylation of
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**FIGURE 9.** Aur1p is not required for the biosynthesis of mannosylated PI*. A–D, before labeling, WT and pmi40 (A) and pmi40 lcb1Δ SLC1-1 (FBY952) cells were growing exponentially in LM supplemented with 2 mM mannosate at 30 °C. Myriocin (M), 40 μg/ml or AbA (1 μg/ml) were added, and cells were further grown for 100 min. 10 A°/0 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 (J) or [2-3H]mannose (M). D, the preculture additionally contained 5 μM PHS; AbA was present during preculture and labeling (lanes 10 and 23) or only during labeling (lanes 24 and 25). MPIs are marked with an asterisk, and the regions containing mannosyl-PI* (MPI*) are boxed.

IPCs 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 4Δ cells contained negative ions of m/z 1137, which corresponds to the expected m/z of mannosyl-PI4:1 with oleic acid in sn-1 and C26:0 in sn-2, albeit in quantities too low to allow fragmentation (not shown).

**FIGURE 10.** SLC1-1 and IPC mannosyltransferases both contribute to the viability of cells when sphingolipid biosynthesis is blocked. Strains FBY999–FBY9110 were grown to exponential phase, and suspensions of an A°/0 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 tetrasporic 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 cccΔ. 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 \( W4 \Delta \text{SLC1-1} \) 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 \textit{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, \( W4 \Delta \text{SLC1-1} \) 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 \( \text{PHS} \) then without (not shown). Moreover, the overexpression of \( UBI4 \), encoding for ubiquitin, could not rescue the heat sensitivity of \( W4 \Delta \text{SLC1-1} \) 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 proteasomal degradation of aggregated proteins (53). Inspection of the overview in Table 1 shows that only \( W4 \Delta \text{SLC1-1} \) cells grow at 37 °C. This suggests that for cells lacking ceramide and IPCs, both an \( SLC1-1 \)-dependent lipid and significant \( \text{PHS} \) 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 \( W4 \Delta \text{SLC1-1} \) and \( lcb1-100 \) cells. \( W4 \Delta \text{SLC1-1} \) 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 reinitiating heat shock proteins because they are unable to raise LCB levels during heat stress (52). Altogether the data illustrate that even the concomitant presence of large amounts of \( \text{PHS} \), 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 \( lag1 \Delta lac1 \Delta ypc1 \Delta ydc1 \Delta \) 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 \( \text{AbA} \) (Fig. 4B; supplemental Fig. S5) (13), and lyso-IPCs therefore seem to be made by \( \text{Aur1p} \). \( \text{Aur1p} \) has previously been shown to use not only the physiologically 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, \( \text{Aur1p} \) can even use free LCBs, albeit with a preference for LCB-C20 over LCB-C18. However, we could not find ions corresponding to mannosylated lyso-IPCs, suggesting that lyso-IPCs are not a substrate for \( \text{Csg1p} \) or \( \text{Csh1p} \).

This study was triggered by the stunning observation that \( 4 \Delta \) cells continue to add ceramides to GPI anchors (Fig. 4). Comparison of the relative amounts of [\( ^{3} \text{H} \)]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

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**TABLE 1**

heat resistance of sphingolipid biosynthesis mutants

+ = normal; − = absent; NT = not tested.

| Genotype          | Lipids made | Growth at | Reference |
|-------------------|-------------|-----------|-----------|
|                   | LCB | Ceramide | IPCs | PI | 24 °C | 37 °C | Survival at 44 °C |
| **WT**            | +  | +        | +    | −  | +    | +    | +               | This study |
| \( lcb1-1 \) \( \text{SLC1-1} \) | +  | ⊳ ⊳ ⊳ | ⊳ ⊳ | ⊳ ⊳ | +    | − ⊳ | ⊳      | (53) |
| \( lcb1-100 \)   | +  | +        | −    | ⊳ ⊳ | +    | − ⊳ | ⊳      | (13, 25) |
| \( W303.4 \Delta \) | ⊳ ⊳ | +        | +    | −  | + ⊳ | − ⊳ | NT             | (53, 58, 59) |
| \( W4 \Delta \text{SLC1-1} \) | ⊳ ⊳ | +        | +    | −  | + ⊳ | − ⊳ | NT             | This study |

**FIGURE 11.** Heat shock resistance of \( 4 \Delta \text{SLC1-1} \) cells. Two of three representative experiments are shown. \( A \) and \( B \), the indicated strains were pre-cultured at 24 °C in LM and diluted to 0.1 \( A_{600} \) and aliquots were incubated for 0, 2, 5, 12, and 30 min at 44 °C. Serial 3.3-fold dilutions were spotted with a micropipette onto LM plates containing 25 μm PHS. (Reproducible results were obtained only, when cells were not subjected to mechanical stress after the heat shock.)
incorporation of ceramides into GPI anchors. Either the Cwh43p ceramide remodelase has a higher affinity for ceramides than Aur1p, or the ceramides of 4Δ 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 4Δ cells and WT cells is the same, suggesting that the unknown enzyme generating ceramides in 4Δ 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 with shorter fatty acids that are generated in 4Δ.Lass5 cells (supplemental Fig. S4) nor non-acylated LCBs. On the other hand, as stated before, Aur1p accepts ceramides with C20:0 down to C2:0 fatty acids as substrates (13, 29, 56). Thus, if the unknown ceramide synthase of 4Δ cells would make ceramides with shorter fatty acids, they should be transformed into IPCs. As this is not the case, we can assume that the unknown ceramide synthase of 4Δ cells is specific for very long chain fatty acids.

Aureobasidin A at 2.5 μg/ml stops the growth of W303.4Δ cells (13), whereas it slows, but does not stop, the growth of W4Δ.SLC1-1 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 4Δ.SLC1-1 cells must be achieved through blocking the synthesis of IPCs and/or lyso-IPCs, 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 4Δ cells. The data therefore suggest that lyso-IPCs and/or IPCs make an essential contribution to the cell viability of W4Δ.SLC1-1 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 lcb1Δ SLC1-1 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 laciΔ lag1Δ cells (26, 29) (Fig. 6C). However, it seems that PI" is not able to rescue lcb1Δ or YPK9.4Δ cells, but only PI", carrying a C26:0 in sn-2. It may be speculated that the PI" fails to rescue lcb1Δ or YPK9.4Δ 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 YPK9.4Δ cells as on lcb1Δ cells, we could not directly confirm that mannosylation of PI" is also crucial for the survival of 4Δ cells because 4Δ 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 4Δ cells.

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