

# C26-CoA-dependent ceramide synthesis of *Saccharomyces cerevisiae* is operated by Lag1p and Lac1p

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**Lag1p and Lac1p are two highly homologous membrane proteins of the endoplasmic reticulum (ER). When both genes are deleted, cells cannot transport glycosylphosphatidylinositol (GPI)-anchored proteins from the ER to the Golgi at a normal rate. Here we show that microsomes or detergent extracts from *lag1Δlac1Δ* double mutants lack an activity transferring C26 fatty acids from C26-coenzyme A onto dihydrosphingosine or phytosphingosine. As a consequence, in intact cells, the normal ceramides and inositolphosphorylceramides are drastically reduced. *lag1Δlac1Δ* cells compensate for the lack of normal sphingolipids by making increased amounts of C26 fatty acids, which become incorporated into glycerophospholipids. They also contain 20- to 25-fold more free long chain bases than wild type and accumulate very large amounts of abnormally polar ceramides. They make small amounts of abnormal mild base-resistant inositolphospholipids. The lipid remodelling of GPI-anchored proteins is severely compromised in *lag1Δlac1Δ* double mutants since only few and mostly abnormal ceramides are incorporated into the GPI anchors. The participation of Lag1p and Lac1p in ceramide synthesis may explain their role in determining longevity.**

**Keywords:** fumonisin/glycosylphosphatidylinositol/lipid remodelling/longevity genes/sphingolipids

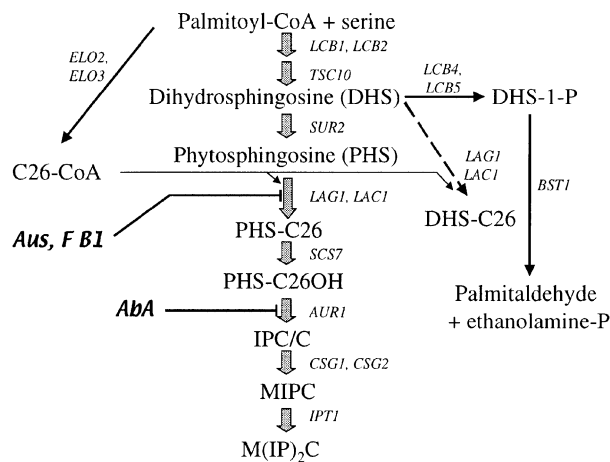
## Introduction

Sphingolipids are a complex class of membrane lipids present in all eukaryotes. Essential features of the membrane architecture depend on the presence of sphingolipids, which seem to be able to aggregate laterally and form specialized areas in the membrane, which are referred to as rafts or microdomains.

The only abundant sphingolipids of *Saccharomyces cerevisiae* are the inositolphosphorylceramides (IPCs), mannosyl-IPCs (MIPCs) and inositolphosphoryl-MIPC [M(IP)<sub>2</sub>C] (Figure 1). Cells that cannot make IPCs are non-viable, whereas mannosylation of IPCs is dispensable (Beeler *et al.*, 1997; Nagiec *et al.*, 1997). Beyond their

structural role, sphingolipids of yeast have also been proposed to be signals for adaptive responses to heat and other stresses. Indeed, shifting cells from 24 to 37°C causes transient increases in the levels of dihydrosphingosine (DHS), phytosphingosine (PHS), PHS-1 phosphate (PHS-1-P), DHS-1-P and a sustained elevation of free ceramides (Dickson *et al.*, 1997b; Jenkins *et al.*, 1997; Skrzypek *et al.*, 1999). The elevation of ceramide levels is inhibited by australifungin (Aus) or fumonisin B1, two well-known inhibitors of ceramide synthase, thus suggesting that heat shock induces *de novo* synthesis of these signalling molecules (Jenkins *et al.*, 1997; Wells *et al.*, 1998). Several mutants accumulating DHS-1-P and PHS-1-P survive better at elevated temperature than wild-type cells, suggesting that such compounds induce resistance to heat stress (Mandala *et al.*, 1998; Mao *et al.*, 1999; Skrzypek *et al.*, 1999). It has also been proposed that the extension of the yeast replicative life span afforded by transient, sublethal heat stress and the dependence of this phenomenon on *RAS1* (Shama *et al.*, 1998) are caused by alterations in sphingolipid metabolism (Jazwinski, 1999). The rapid ubiquitylation and degradation of the surface permease Fur4p after heat stress also seem to be mediated through a heat-induced sphingolipid (Chung *et al.*, 2000). Moreover, ceramide addition to yeast cells leads to a G<sub>1</sub> cell cycle arrest, and this effect is mediated through a ceramide-activated protein phosphatase (Fishbein *et al.*, 1993; Nickels and Broach, 1996).

A further well-documented role of sphingolipid metabolites in yeast concerns their involvement in the vesicular transport of glycosylphosphatidylinositol (GPI)-anchored proteins out of the endoplasmic reticulum (ER) (Horvath *et al.*, 1994; Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997) and in endocytosis (Zanolari *et al.*, 2000). Transport of GPI-anchored proteins out of the ER in living cells is severely retarded if one reduces, either pharmacologically or genetically, the activity of the serine palmitoyltransferase (*LCB1*, *LCB2*), which is the key enzyme of DHS biosynthesis. It appears that the ER to Golgi transport of GPI-anchored proteins requires several dedicated components, which are dispensable for the transport of soluble proteins and ordinary membrane proteins with classical membrane-spanning domains. Dedicated components comprise sphingolipids, the cargo receptor Emp24p, components of the Golgi to ER retrotransport machinery and, as recently reported, Lag1p or Lac1p (Schimmöller *et al.*, 1995; Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997; Barz and Walter, 1999; Muniz *et al.*, 2000). Indeed, *lag1Δlac1Δ* cells transport GPI-anchored proteins from the ER to the Golgi at a reduced rate (Barz and Walter, 1999). Attention was drawn first to *LAG1* since a gene deletion in haploid cells resulted in a pronounced increase (~50%) in mean and maximum life span (D'mello *et al.*, 1994). *LAG1* has a close homologue in yeast, which



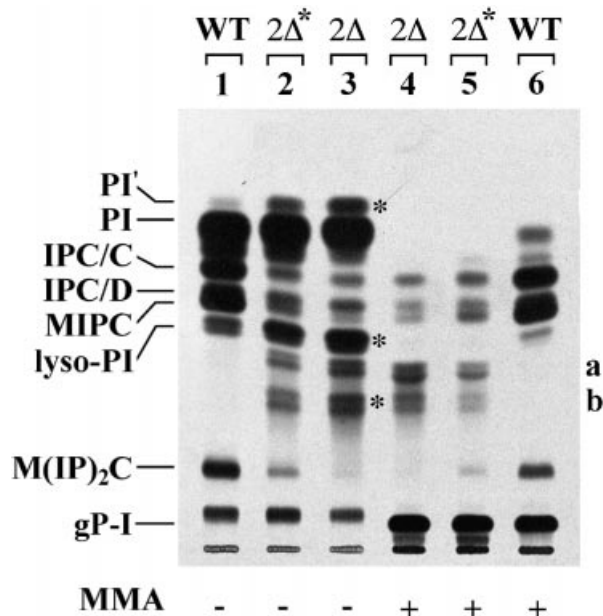
**Fig. 1.** A simplified scheme of sphingolipid biosynthesis in *S. cerevisiae*. The dashed arrow indicates the reaction observed in microsomes (see Figures 4 and 5). Aus, australifungin; AbA, aureobasidin A; F B1, fumonisin B1.

was called *LAC1* (*DGT1*), and orthologues of these two genes occur in a wide variety of eukaryotic cells (Jiang *et al.*, 1998; Brandwagt *et al.*, 2000). *LAG1* and *LAC1* have significant sequence similarity to TRAM, a mammalian membrane protein thought to be involved in protein translocation across the ER membrane, but no translocation defects were detected in *lag1Δlac1Δ* yeast cells (Barz and Walter, 1999). Furthermore, human TRAM does not complement the growth defect in a *lag1Δlac1Δ* double mutant, in contrast to another human gene called *LAG1Hs* (Jiang *et al.*, 1998). While testing *lag1Δlac1Δ* cells for their capacity to remodel the lipid moiety of the GPI anchors, we realized that they have a severe remodelling defect, which is due to a general deficiency in ceramide biosynthesis.

## Results

### **Deletion of *LAG1* and *LAC1* leads to drastically reduced amounts of IPCs**

Whereas the single deletion of *LAG1* or *LAC1* has no phenotype, the concomitant deletion of *LAG1* and *LAC1* has been reported to cause a severe growth defect in the genetic background of W303 cells (Barz and Walter, 1999). The same double deletion did not yield viable cells in the background of YPK9 cells (Jiang *et al.*, 1998). In our hands, *lag1Δlac1Δ* mutants generated in the W303 background grew only if the media were supplemented with osmotic stabilizers and high vitamin concentrations. However, they tended to adapt and to become overgrown by suppressors. To analyse the function of *LAG1* and *LAC1*, we therefore used either short-term cultures of W303-1A *lag1Δlac1Δ* (designated 2Δ) or YPK9 *lag1Δlac1Δ* pBM150:*LAG1* containing a copy of *LAG1* under the control of the *GALI* promoter. The latter strain has a wild-type growth phenotype when grown on galactose, but ceases to grow within 30 h when shifted to glucose, and at that stage is designated 2Δ\*. Figure 2 shows one of many experiments in which the lipid extracts of [<sup>3</sup>H]inositol-labelled double mutants were compared with wild-type

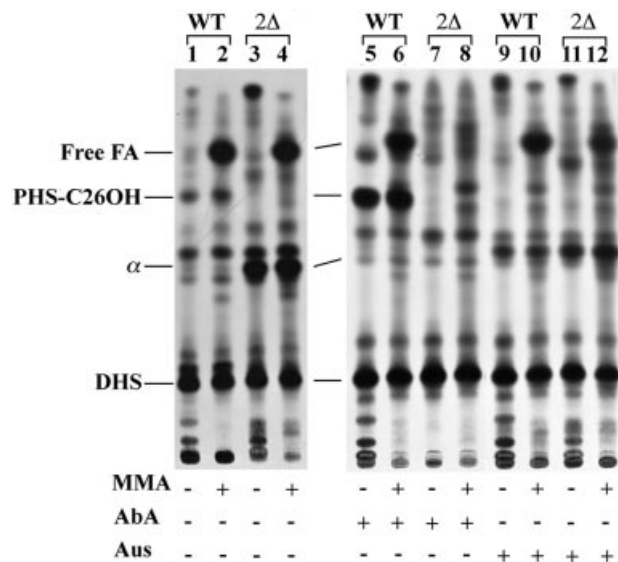


**Fig. 2.** Deletion of *LAG1* and *LAC1* strongly reduces the amounts of IPCs. Ten OD of W303-1A (WT), YPK9 *lag1Δlac1Δ* harbouring pBM150:*LAG1* (2Δ\*), depleted of *Lag1p* by pre-culture on glucose for 30 h, and W303-1A *lag1Δlac1Δ* (2Δ) strains were radiolabelled with [<sup>3</sup>H]inositol at 30°C. The lipids were extracted, desalted, treated with monomethylamine (MMA) for saponification as indicated, and analysed on TLC using solvent system 2. Glycerolphosphorylinositol (gP-I) visible in lanes 4–6 was generated by saponification. The asterisks mark some abnormal lipids of 2Δ\* and 2Δ.

extracts. In all experiments, we found a very significant reduction in the classical structural sphingolipids IPC/C, IPC/D, MIPC and M(IP)<sub>2</sub>C. Quantitation of this experiment shows that the sum of these sphingolipids accounts for 26, 6 and 3% of total inositol-containing phospholipids in wild-type, 2Δ\* and 2Δ cells, respectively. Thus, structural sphingolipids are all significantly decreased, and 2Δ cells seem to be more severely affected than 2Δ\* cells. Moreover, 2Δ\* and 2Δ cells make several mild base-resistant [<sup>3</sup>H]inositol-labelled lipids (Figure 2, lanes 2–5), which run between MIPC and M(IP)<sub>2</sub>C and which cannot be detected in wild-type cells (Figure 2, lanes 1 and 6). In 2Δ and 2Δ\* cells, the sum of these abnormal lipids (a and b) accounts for 62 and 35% of total mild base-resistant lipids, respectively. We speculate that they represent IPCs having shorter fatty acids (FAs) than the normal IPCs (see below). Another abnormality of the lipid profile of 2Δ\* and 2Δ cells is the presence of a distinct, mild base-sensitive band above phosphatidylinositol (PI; band labeled PI' in Figure 2). Moreover, 2Δ\* and 2Δ cells contain a major band co-migrating with lyso-PI, which is abnormal, since it is clearly resolved from lyso-PI when other solvent systems are used (not shown).

### ***lag1Δlac1Δ* cells do not make PHS-C26OH**

Ceramide biosynthesis was assayed by labelling cells with [<sup>3</sup>H]DHS, which is an obligatory intermediate in the biosynthesis of ceramide. Exogenously added [<sup>3</sup>H]DHS is readily incorporated into all sphingolipids (Reggiori *et al.*, 1997). We reasoned that the *lag1Δlac1Δ* mutant should incorporate this physiological intermediate into sphingolipids normally if the deficiency of *lag1Δlac1Δ* mutants



**Fig. 3.** The *lag1Δlac1Δ* cells cannot make PHS-C26OH. Two and a half OD of W303-1A (WT) and W303-1A *lag1Δlac1Δ* (2Δ) strains were pre-incubated with Aus, AbA or solvent for 10 min and were radiolabelled with 25  $\mu$ Ci of [ $^3$ H]DHS at 30°C for 1 h. Lipid extracts were then *O*-deacylated with MMA or control incubated and analysed by TLC in solvent system 1.

was due to a problem in the synthesis of DHS. As can be seen from Figure 3, [ $^3$ H]DHS labelling of intact cells showed that 2Δ did not make any PHS-C26OH, a ceramide having a hydroxylated FA and which corresponds to the ceramide moiety of IPC/C, the main IPC in *S.cerevisiae* (Reggiori *et al.*, 1997). The identity of this band was established through strong acid hydrolysis, yielding [ $^3$ H]PHS, and comparison with a standard obtained through PI-phospholipase C (PLC) treatment of IPC/C (not shown). The absence of PHS-C26OH in 2Δ cells is not just due to an increased rate of utilization of this intermediate, since PHS-C26OH is also undetectable if its incorporation into IPCs is blocked by the addition of aureobasidin A (AbA), a specific inhibitor of the PI ceramide phosphorylinositol transferase *AUR1* (Nagiec *et al.*, 1997). While this drug strongly increases the relative amount of PHS-C26OH in wild-type cells, there is no appearance of this intermediate in 2Δ cells (Figure 3, compare lanes 5 and 7). On the other hand, as expected, the generation of PHS-C26OH is abrogated in the presence of Aus, a specific inhibitor of ceramide synthase (Mandala *et al.*, 1995), which transfers FAs from acyl-CoA to DHS or PHS (Figure 3, lanes 9 and 11). In summary, the absence of IPCs in 2Δ cells can best be explained by a specific biosynthetic defect in the step transforming DHS into ceramide. The defect is not due to a general inability to utilize exogenous DHS since DHS is incorporated into glycerophospholipids efficiently through a pathway that requires phosphorylation to DHS-1-phosphate by *LCB4* or *LCB5* and cleavage to palmitaldehyde plus ethanolamine phosphate through the action of *BST1/DPL1* (Saba *et al.*, 1997; Nagiec *et al.*, 1998) (Figure 1). Indeed, a prominent hydrophobic spot appearing only after mild base treatment of lipid extracts in wild-type as well as in 2Δ cells indicates the liberation of free [ $^3$ H]FAs (Figure 3, lanes 1–4). Interestingly, *lag1Δlac1Δ* mutants make large amounts of

an abnormal mild base-resistant lipid, lipid  $\alpha$ , which may compensate for the loss of PHS-C26OH (Figure 3, lanes 3 and 4). The same lipid is also made by wild-type cells treated with Aus (Figure 3, lanes 9–12). Thus, the pharmacological inhibition of ceramide synthase not only abrogates the accumulation of PHS-C26OH, but also induces the appearance of lipid  $\alpha$ , thus reproducing the lipid abnormalities of *lag1Δlac1Δ* mutants. While lipid  $\alpha$  is resistant to mild base, it yields [ $^3$ H]DHS upon strong acid hydrolysis (not shown), suggesting that it is some kind of ceramide. The fact that it is not made in the presence of AbA is presently not explained.

#### **The *lag1Δlac1Δ* cells up-regulate the biosynthesis of very long chain fatty acids**

The absence of PHS-C26OH biosynthesis cannot be explained by deficiencies in the hydroxylation of DHS to PHS or of C26 to C26OH, since mutants defective in these hydroxylation steps have no problem in making IPCs (Haak *et al.*, 1997). Thus, *lag1Δlac1Δ* mutants must either have a defect in the ceramide synthase or be deficient in making acyl-CoA. Since, in general, IPCs of *S.cerevisiae* contain C26 or C24 FAs or mono- or di-hydroxylated derivatives thereof, we undertook to investigate whether *lag1Δlac1Δ* mutants can elongate C16-CoA to C26-CoA using Elo2p, Elo3p and Tsc13p (Oh *et al.*, 1997; Kohlwein *et al.*, 2001). We thus analysed the content of very long chain FAs of *lag1Δlac1Δ* mutants by gas chromatography-mass spectroscopy (GC-MS). For this, the lipid extracts of cells were treated with methanolic boron fluoride (BF<sub>3</sub>) under conditions in which FAs esterified to glycerophospholipids are liberated and are methylated together with free FAs. As can be seen from Table I, in 2Δ cells the relative percentages of FAs up to C20 are not grossly altered as compared with wild type, but 2Δ cells show a discrete elevation of C20–C24 FAs and have a drastic increase in C26. After strong acid hydrolysis, which also liberates the FAs of sphingolipids, we still observed a very significant increase of C26:0 in 2Δ cells relative to wild type. Overall, it is clear that 2Δ cells are perfectly able to elongate FAs. Moreover, it seems that the lack of Lag1p and Lac1p induces the elongation pathway or inhibits the degradation pathway such as to generate an at least 4-fold higher level of C26:0 than normal. The massive excess of C26:0 is probably incorporated into glycerophospholipids, thus allowing the cells to generate C26:0-containing membrane lipids that may be able to take over some of the functions of sphingolipids, which, in normal cells, are the only lipids containing these very long chain FAs. This may explain the appearance of the [ $^3$ H]inositol-labelled, mild base-sensitive lipid PI' in Figure 2, lane 3. Indeed, preliminary experiments indicate that this lipid is a PI with abnormally long FAs (not shown).

#### **Microsomes of *lag1Δlac1Δ* mutants lack ceramide synthase activity**

The results presented above persuaded us that the problem of *lag1Δlac1Δ* mutants was due to a problem with the ceramide synthase itself. Therefore, we measured ceramide synthase activity in microsomes by adding [ $^3$ H]DHS and acyl-CoA with FAs of different chain length (Mandala *et al.*, 1995). As shown in Figure 4A, lipid

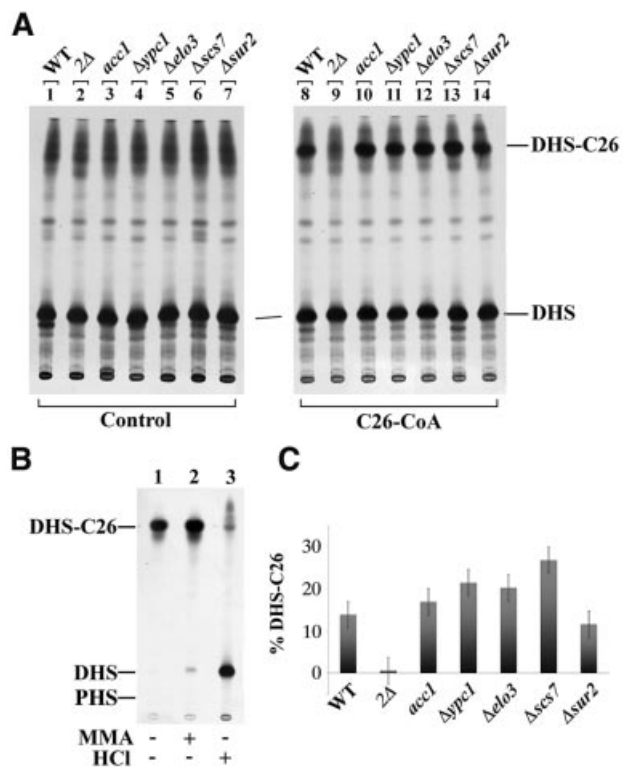
**Table 1.** *lag1Δlac1Δ* cells strongly up-regulate the biosynthesis of very long chain fatty acids<sup>a</sup>

	WT	2Δ	WT + HCl	2Δ + HCl
C14:1	0.235 ± 0.002	0.159 ± 0.061	0.127	0.046
C14:0	1.261 ± 0.159	0.567 ± 0.149	0.831	0.419
C16:1	23.59 ± 0.357	21.56 ± 0.387	25.31	25.07
C16:0	18.89 ± 0.283	11.91 ± 0.520	16.2	9.248
C18:1	38.73 ± 0.233	33.22 ± 0.548	44.74	43.65
C18:0	14.81 ± 1.381	24.12 ± 2.155	10.22	14.68
C20:1	0 ± 0.000	0 ± 0.000	0.332	0.544
C20:0	0.778 ± 0.230	1.53 ± 0.301	0.291	0.474
C22:1	0.479 ± 0.045	0.547 ± 0.086	0.369	0.966
C22:0	0.065 ± 0.065	0.288 ± 0.162	0.143	0.178
C24:1	0.438 ± 0.094	0.444 ± 0.107	0.271	0.691
C24:0	0.144 ± 0.144	0.447 ± 0.099	0.209	0.357
C26:1	0.038 ± 0.038	0.123 ± 0.123	0	0
C26:0	0.266 ± 1.434	4.886 ± 0.037	0.892	3.653

<sup>a</sup>The lipids of W303-1A and W303-1A *lag1Δlac1Δ* strains were methylated either directly (columns WT and 2Δ) or after strong acid hydrolysis (columns WT + HCl and 2Δ + HCl) and analysed by GC-MS as described in Materials and methods. C16, C18 and C26 fatty acids were used as standards. Numbers indicate the percentage of a given fatty acid species compared with the total of identified peaks in the gas chromatogram. With the BF<sub>3</sub> methylation procedure, we were not able to identify the hydroxylated fatty acids, since we had no appropriate standards and the conditions for their quantitative methylation had not been worked out. Thus, only 80% of the signals of the GC profile could be accounted for. Standard deviations represent variations obtained in two independent experiments. The same results as shown in columns WT and 2Δ were obtained when lipid extracts were treated with MMA before being methylated with methanolic BF<sub>3</sub>, indicating that the release of fatty acids from glycerophospholipids was complete (not shown).

extracts of labelled microsomes contained lipids migrating with high mobility typical of ceramides or other unipolar compounds. In the absence of acyl-CoA, we observed a relatively diffuse smear (lanes 1–7), which was already present in the [<sup>3</sup>H]DHS that was added to microsomes (not shown), since the radiolabelled [<sup>3</sup>H]DHS is quite unstable and rapidly generates hydrophobic breakdown products after preparative purification by TLC (Lynch, 2000). However, when C26-CoA was added, a distinct band was generated on top of this material by microsomes from wild-type but not from *lag1Δlac1Δ* cells (Figure 4A). This band was assumed to be DHS-C26, based on the following indirect evidence: (i) the lipid is only generated when C26-CoA is added to the incubations, suggesting that it contains C26; (ii) the band resisted mild base but yielded [<sup>3</sup>H]DHS with strong acid (Figure 4B); (iii) as seen in Figure 5A, biosynthesis of this lipid in microsomes was blocked by the classical ceramide synthase inhibitors Aus and fumonisin B1 (Wu *et al.*, 1995); and (iv) the FA was probably not hydroxylated since in *scs7Δ* cells, which are deficient in  $\alpha$ -hydroxylation of FAs (Dunn *et al.*, 1998), the band had the same mobility as in wild type (Figure 4A, lane 13; Figure 1). The band was also not generated when free C26, C16-CoA or free C16 were added in the presence of ATP and CoA (Figure 5C) or in the presence of C20-CoA (not shown). This result strongly indicates that the activity we observe requires a CoA-activated very long chain FA.

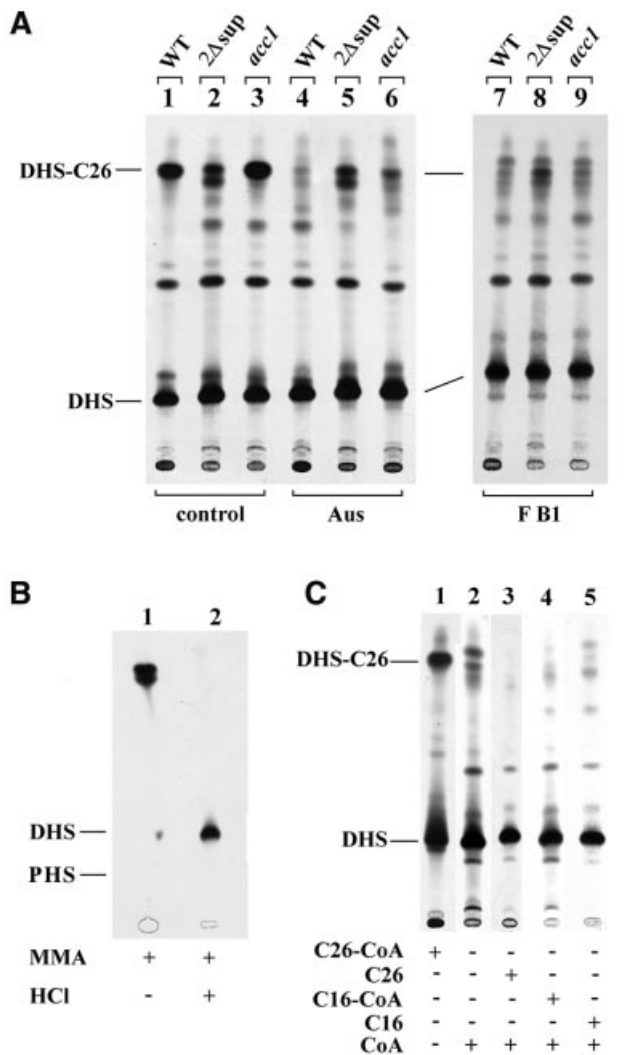
Microsomes from *accl* and *elo3Δ* (deficient in FA elongation; Schneiter *et al.*, 1996; Oh *et al.*, 1997), from *ypc1Δ* (deficient in alkaline ceramidase; Mao *et al.*, 2000)



**Fig. 4.** Microsomes of *lag1Δlac1Δ* mutants lack DHS-C26 synthase activity. (A) Microsomes of W303-1A (WT), W303-1A *lag1Δlac1Δ* (2Δ) and the other mutants indicated at the top were radiolabelled with [<sup>3</sup>H]DHS without (Control) or in the presence of C26-CoA (0.2 mM). The thermosensitive *accl* cells were pre-incubated for 2 h at 37°C before preparing microsomes. The radiolabelled lipids were extracted, treated with MMA and analysed by TLC developed in solvent system 1. (B) The band labelled DHS-C26 in (A) was purified by two successive rounds of preparative TLC. The extract was divided into three aliquots, one of which remained untreated, one was MMA treated and the third was treated with MMA followed by strong acid hydrolysis. The three samples were then analysed by TLC developed in solvent system 1. (C) The amount of radioactivity in the spots of DHS-C26 of (A) lanes 8–14 was quantified by radioscanning and the results were expressed as the percentage of counts in [<sup>3</sup>H]DHS-C26 as compared with total radioactivity in that lane. Error bars indicate standard deviations in two entirely independent experiments.

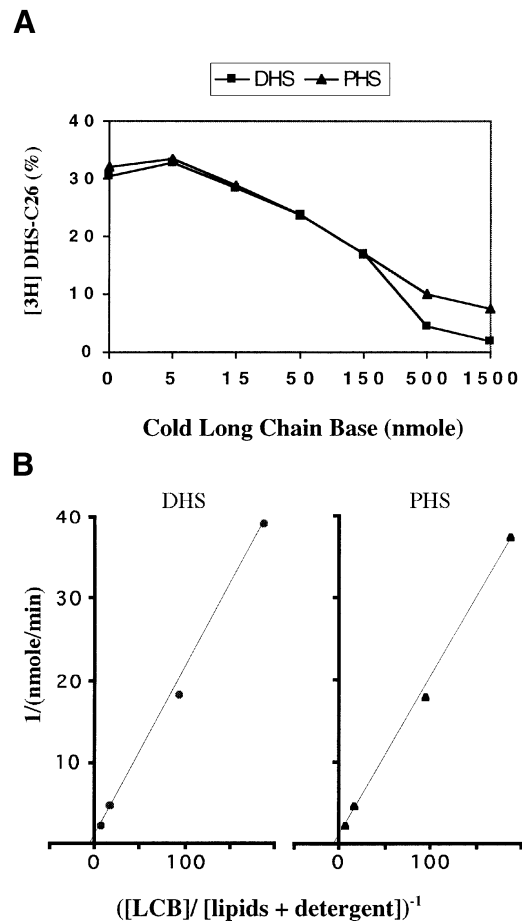
and from *sur2Δ* (deficient in the generation of PHS; Haak *et al.*, 1997; Grilley *et al.*, 1998) all contain normal or elevated ceramide synthase activity, as judged by this microsomal assay (Figures 1 and 4C). Thus, we conclude that microsomes of *lag1Δlac1Δ* lack a C26-CoA-dependent ceramide synthase activity *in vitro*. This is concordant with the absence of PHS-C26OH observed in lipid extracts from [<sup>3</sup>H]DHS-labelled cells (Figure 3) except that the microsomal *in vitro* system apparently does not support hydroxylation of DHS or C26:0, a feature that is not unexpected, since the microsomal system did not contain any NADPH.

The microsomal activity generating DHS-C26 in a C26-CoA- and Lag1p/Lac1p-dependent manner could be solubilized efficiently by incubation in 1% digitonin at 4°C for 2 h with a recovery of activity of >100%. This allowed us to perform the assay in the presence of various amounts of cold DHS or PHS as shown in Figure 6A. Cold DHS and PHS both inhibit incorporation of [<sup>3</sup>H]DHS into [<sup>3</sup>H]DHS-C26. DHS, by definition, acts as a competitive inhibitor of [<sup>3</sup>H]DHS incorporation. In the physiological



**Fig. 5.** Biosynthesis of DHS-C26 is sensitive to Aus and fumonisin B1 (F B1). (A) Microsomes of W303-1A, W303-1A *lag1Δlac1Δ* containing a suppressor mutation ( $2\Delta^{sup}$ ) and *acc1* were labelled with [ $^3$ H]DHS in the presence of C26-CoA (0.2 mM). Microsomes were pre-incubated for 10 min with Aus, F B1 or without inhibitor (control). Lipids were extracted, MMA treated and analysed by TLC in solvent system 1. (B) The MMA-treated DHS-C26-like lipid from  $2\Delta^{sup}$  was purified by two successive rounds of preparative TLC and treated with strong HCl before being analysed by TLC with solvent system 1. (C) Microsomes of W303-1A cells were labelled with [ $^3$ H]DHS under standard conditions (Materials and methods) but in the presence of C26-CoA, C26, C16-CoA, C16 and CoA as indicated.

concentration range, PHS and DHS have overlapping inhibition curves, strongly suggesting that PHS is also a substrate which has about the same affinity for the enzyme as DHS. At long chain base/carrier lipid ratios of 0.53 and more, DHS was more inhibitory than PHS, but at these concentrations it is questionable whether the inhibition is still only competitive and whether the substrates are solubilized adequately in detergent micelles. From the data obtained using 5–155 nmol of DHS or PHS, we calculated the absolute amounts of product made per minute (Figure 6B) and thus obtained the Lineweaver–Burk plots shown in Figure 6B. They indicated that the apparent  $V_{max}$  values were 1.35 nmol/min for DHS and 1.05 nmol/min for PHS. Half-maximal

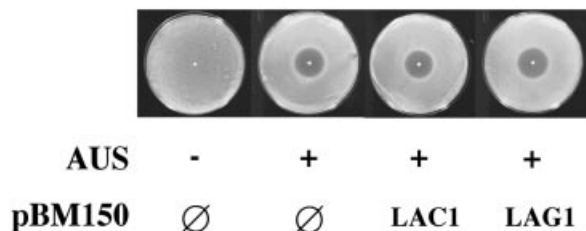


**Fig. 6.**  $K_m$  and  $V_{max}$  of microsomal DHS-C26 synthase activity. (A) Microsomes were lysed in 1% digitonin. A 100  $\mu$ g aliquot of solubilized microsomal extract was assayed in the presence of 5 nmol of [ $^3$ H]DHS, 200 nmol of C26-CoA and variable amounts (5–1500 nmol) of cold DHS or PHS in the presence of 1% digitonin. The products were extracted and analysed by TLC in solvent 1. [ $^3$ H]DHS-C26 was quantitated by radioscanning and expressed as a percentage of total radioactivity in the corresponding lane. (B) The data of (A) were used to calculate enzymatic activities by calculating the total amounts of DHS-C26 made during incubations. DHS or PHS concentrations of the assays were expressed as mol/mol ratios of [long chain bases]/[total phospholipids + detergent]. The data were then transformed in double reciprocal plots according to Lineweaver–Burk. [Assays contained 120 nmol of phospholipids (estimated based on the amount of microsomal protein) and 820 nmol of detergent.]

velocity was reached when the [long chain base]/[cellular lipid + detergent] ratio was 0.27 for DHS and 0.2 for PHS. Thus, it appears that Lag1p/Lac1p can utilize both DHS and PHS. Overexpression of Lag1p or Lac1p under the control of the *GAL1* promoter did not increase the resistance of wild-type cells to Aus (Figure 7). These data are compatible with the hypothesis that a further subunit is required for ceramide synthase activity.

#### Characterization of a suppressor growing in the absence of LAG1 and LAC1

Prolonged culture of *lag1Δlac1Δ* led to the appearance of spontaneous suppressors. One suppressor, labelled  $2\Delta^{sup}$ , was also assayed for microsomal ceramide synthase. As can be seen in Figure 5, lanes 2, 5 and 8, the suppressor's microsomes had regained an activity generating lipids



**Fig. 7.** Overexpression of Lag1p does not confer resistance to Aus. YPK9 cells containing pBM150 either without an insert or harbouring *LAC1* or *LAG1* under the control of the *GAL1* promoter were cultured on yeast nitrogen base with 2% galactose but lacking uracil, and were seeded at 5500 cells per plate containing the same medium. Then a filter paper with either solvent alone or 20  $\mu$ g of Aus was placed in the centre. Plates were photographed after 3 days of incubation at 24°C.

migrating close to DHS-C26, an activity that was insensitive to Aus and also partially resistant to fumonisin B1. These lipids are resistant to mild base and yield [ $^3$ H]DHS upon strong acid hydrolysis (Figure 5B). This suggests that these lipids are DHS-containing ceramides. They are clearly different from the mild base-resistant lipid  $\alpha$  observed in intact cells treated with Aus or in *lag1 $\Delta$ lac1 $\Delta$*  (Figure 3, lanes 3, 4 and 9–12) since the lipids made by the suppressor co-migrate with DHS-C26, whereas lipid  $\alpha$  is more polar than PHS-C26OH, a compound which itself is considerably more polar than DHS-C26. It is conceivable that the activity induced in this suppressor is mediated by Ypc1p, an alkaline ceramidase that can catalyse the reverse reaction, namely the condensation of free FAs with DHS or PHS (Mao *et al.*, 2000). Ypc1p is not blocked by fumonisin B1 and apparently is used to make ceramide when cells are treated with ceramide synthase inhibitors. The acyl-DHS of 2 $\Delta$ <sup>sup</sup> can apparently functionally replace the normal ceramides as substrates for IPC biosynthesis, since when we labelled intact 2 $\Delta$ <sup>sup</sup> cells with [ $^3$ H]DHS or [ $^3$ H]inositol, they exhibited a normal profile of sphingolipids (not shown).

#### ***lag1 $\Delta$ lac1 $\Delta$* cells accumulate free long chain bases and ceramides**

It has been found previously that the treatment of yeast cells with fumonisin B1 increased the cellular concentration of DHS and PHS up to 50-fold (Wu *et al.*, 1995). Unpolar lipids were extracted from wild type and *lag1 $\Delta$ lac1 $\Delta$*  mutants, and free long chain bases were derivatized and quantitated using HPLC. As shown in Table II, while the concentrations of DHS and PHS in wild-type cells were very similar to values reported in the literature (Dickson *et al.*, 1997b), in *lag1 $\Delta$ lac1 $\Delta$*  they were 41- and 16-fold higher. Concentrations of free ceramides were measured after separating them from free long chain bases and IPCs by silica gel chromatography. As seen in Table II, while free ceramide levels in wild-type cells were in the same range as the values reported in the literature (Dickson *et al.*, 1997b), those in *lag1 $\Delta$ lac1 $\Delta$*  were drastically increased. The increase was ~195-fold for DHS-containing ceramides and 21-fold for PHS-containing ceramides. The ceramide levels of *lag1 $\Delta$ lac1 $\Delta$*  are in the range of total inositol-containing sphingolipids found in wild-type cells, i.e. ~750 pmol/OD<sub>600</sub> of cells (Hanson and Lester, 1980). Our procedure did not allow the

**Table II.** Free long chain base and ceramide content of *lag1 $\Delta$ lac1 $\Delta$* <sup>a</sup>

	W303-1A	W303-1A <i>lag1<math>\Delta</math>lac1<math>\Delta</math></i>
Free DHS	10 $\pm$ 3.7 <sup>b</sup>	401 $\pm$ 81
Free PHS	28 $\pm$ 7.2	454 $\pm$ 152
DHS in Cer	4 $\pm$ 1.7	974 $\pm$ 398
PHS in Cer	30 $\pm$ 7.0	660 $\pm$ 277

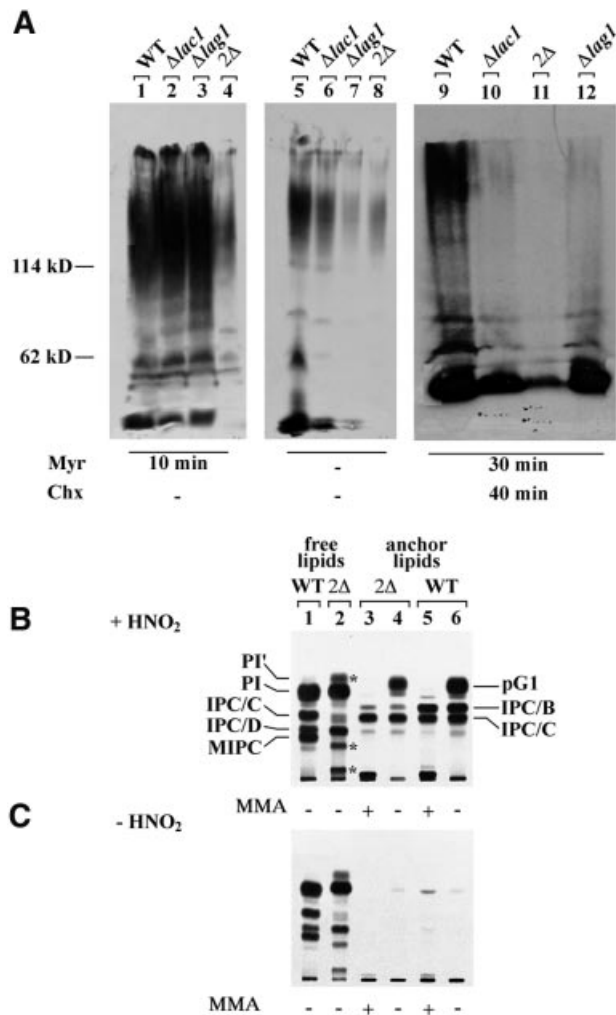
<sup>a</sup>All results are given as pmol/OD<sub>600</sub> unit of cells.

<sup>b</sup>Results are averaged from five independent experiments performed on different days and with different cell cultures.

identification of the FA of the ceramides, but it seems likely that these abundant free ceramides of *lag1 $\Delta$ lac1 $\Delta$*  consist of lipid  $\alpha$  (Figure 3, lanes 3 and 4), which most probably represents an abnormally polar ceramide (see above). We interpret the rise in ceramides in the sense that the lack of Lag1p and Lac1p induces the DHS levels to rise sufficiently to permit some unknown enzyme(s) to catalyse the condensation of DHS or PHS with component X, thus generating lipid  $\alpha$ . Whatever the exact nature of ceramides accumulating in *lag1 $\Delta$ lac1 $\Delta$* , it would appear that they are bad substrates for the IPC synthase Aur1p (Nagiec *et al.*, 1997) or that they have difficulty in accessing the compartment in which Aur1p is active. In view of the huge accumulation of abnormal ceramides, one might consider that Lag1p and Lac1p are not catalytic subunits of the ceramide synthase, but rather are required to make it specific for C26-CoA. However, this is ruled out by the fact that microsomes from neither wild type (Figure 5C, lane 4) nor *lag1 $\Delta$ lac1 $\Delta$*  (not shown) synthesize any ceramides in the presence of C16-CoA.

#### ***lag1 $\Delta$ lac1 $\Delta$* utilizes abnormal ceramides to remodel the lipid moiety of GPI anchors**

The PI moiety of the GPI added to newly made GPI-anchored proteins in the ER is indistinguishable from ordinary PI and probably contains C16 or C18 FAs. This PI moiety, however, is remodelled rapidly so that more mature GPI proteins either possess a diacylglycerol with a C26 FA in the *sn*-2 position or contain a ceramide, namely PHS-C26 or PHS-C26OH. The latter ceramide is only found in GPI-anchored proteins that have reached the Golgi apparatus (Reggiori *et al.*, 1997; Sipos *et al.*, 1997). Since it has been observed that Gas1p and Yap3p, two well-characterized GPI-anchored proteins of yeast, are not transported out of the ER with normal kinetics in *lag1 $\Delta$ lac1 $\Delta$*  (Barz and Walter, 1999), it was of interest to see whether they remodelled GPI anchors normally. Metabolic labelling of cells with [ $^3$ H]DHS results in the labelling of a number of highly and very heterogeneously glycosylated proteins, which are all GPI-anchored proteins (Reggiori *et al.*, 1997). In normal cells, most GPI-anchored proteins have ceramide-based anchors. A normal incorporation of [ $^3$ H]DHS into proteins thus indicates that a cell makes normal amounts of GPI-anchored proteins and has no problem in introducing the ceramide moiety into GPI anchors. As can be seen in Figure 8A, the incorporation of [ $^3$ H]DHS into proteins was diminished in *lag1 $\Delta$* , *lac1 $\Delta$*  and *lag1 $\Delta$ lac1 $\Delta$*  (lanes 5–8), but could be normalized in the single deletants by the addition of myriocin, a specific inhibitor of serine palmitoyltransferase, thus blocking the endogenous biosynthesis of DHS



**Fig. 8.** *lag1Δlac1Δ* utilizes abnormal ceramides to remodel the lipid moiety of GPI anchors. (A) Two and a half OD of W303-1A (WT), W303-1A *lac1Δ*, W303-1A *lag1Δ* and W303-1A *lag1Δlac1Δ* were radiolabelled at 30°C with 25  $\mu$ Ci of [ $^3$ H]DHS, and proteins were analysed by SDS-PAGE/fluorography. Before addition of the label, cells were pre-incubated for the indicated times with the inhibitors myriocin (myr) or cycloheximide (Chx). (B and C) Ten OD<sub>600</sub> of W303-1A (WT) and W303-1A *lag1Δlac1Δ* ( $\Delta$ ) were radiolabelled with [ $^3$ H]inositol at 30°C and GPI-anchored peptides free of contaminating lipids were prepared. Anchor peptides were treated with HNO<sub>2</sub> (B) or control incubated (C). The liberated lipids were desalted by butanol extraction and incubated with or without MMA. Anchor lipids (lanes 3–6) together with the free lipids obtained by the delipidation procedure (lanes 1 and 2) were analysed on TLC using solvent system 3. pG1 indicates the typical phosphatidylinositol found on GPI anchors. The asterisks marks the several abnormal lipids of  $\Delta$  already pointed out in Figure 2. The absence of anchor lipids in lanes 3–6 of (C) indicates that the delipidation of GPI-anchored proteins was complete.

(lanes 1–4). Additionally, we tested remodelling reactions occurring beyond the ER by pre-incubating cells for 40 min in the presence of cycloheximide, thus allowing GPI-anchored proteins to leave the ER (Reggiori *et al.*, 1997). This test showed that the remodelling reactions in the Golgi or at the cell surface were drastically reduced in single and double mutants (Figure 8A, lanes 9–12). Overall, the data indicated a relative deficiency in the biosynthesis and/or remodelling of GPI-anchored proteins. It should be noted that this deficiency need not

be attributed to a general slowing down of metabolic processes in these slowly growing cells, since *lac1Δ* or *lag1Δ* grow normally and since the maturation kinetics of CPY and Pho8p were quite normal in *lag1Δlac1Δ* in spite of a strongly reduced growth rate (Barz and Walter, 1999).

For further analysis, we labelled cells with [ $^3$ H]inositol, a metabolic labelling procedure that specifically labels GPI-anchored proteins, irrespective of the type of anchor lipid. The incorporation of [ $^3$ H]inositol into GPI anchors in *lag1Δlac1Δ* was only 28% of that observed in the wild type, while the incorporation into PI was unaffected. After extensive delipidation, the labelled proteins were digested with pronase, thus generating anchor peptides, and their [ $^3$ H]inositol-phosphoryl-lipid moieties were liberated by treatment with nitrous acid (Guillas *et al.*, 2000). The released anchor lipids were analysed by TLC and compared with the cell's free lipids as shown in Figure 8B. Anchors of normal cells contain the previously characterized phosphoinositides pG1, IPC/B and IPC/C (Sipos *et al.*, 1997) (Figure 8B, lane 6). pG1 is a PI with a C26 FA in *sn*-2 of its glycerol moiety and therefore exhibits a higher mobility than the bulk of the cell's free PI. Thus, although the absolute amount of incorporation of [ $^3$ H]inositol into proteins is significantly reduced in the *lag1Δlac1Δ* mutants, the relative amount of diacylglycerol-based anchors (pG1) is normal (55 instead of 52%). A major difference appears in the mild base-resistant ceramide-based anchors. GPI anchors of wild-type cells contain mainly IPC/B and IPC/C, their ceramide moieties being PHS-C26:0 and PHS-C26OH, respectively (Reggiori *et al.*, 1997). In wild-type cells, IPC/B and IPC/C accounted for 68 and 31%, respectively, of base-resistant anchors (quantitation of Figure 8B, lane 5). Anchors from *lag1Δlac1Δ* contained three base-resistant lipids, two of which co-migrated with IPC/B and IPC/C and accounted for 9.5 and 86% of base-resistant anchors, respectively (quantitation of Figure 8B, lane 3). It is thus apparent that the main base-resistant lipid moiety of GPI anchors of *lag1Δlac1Δ* is not IPC/B as in wild-type cells, but rather a less hydrophobic lipid co-migrating with IPC/C. In normal cells, IPC/C is only found in GPI-anchored proteins that have reached the Golgi apparatus. Since *lag1Δlac1Δ* specifically delays the transport of GPI-anchored proteins to the Golgi, the pulse labelling protocol used in Figure 8 should, in principle, lead to a reduced relative amount of IPC/C in the anchor population. However, we observe the opposite. We therefore suspect that, in spite of their co-migration with IPC/B and IPC/C, the base-resistant lipids used for remodelling in *lag1Δlac1Δ* consist of abnormal ceramides having C16 FAs or some other mild-base-resistant lipid, but further studies will have to confirm this hypothesis.

## Discussion

The data presented here indicate that Lag1p and Lac1p are either coding for a catalytic subunit of ceramide synthase or are obligatory activators for the ceramide synthase activity in yeast. Indeed, they fulfil many of the criteria that we would want to see met by any candidate gene coding for ceramide synthase itself. (i) Homologues have been found in mammals, insects, worms, higher fungi and

plants (Jiang *et al.*, 1998; Brandwagt *et al.*, 2000). (ii) They contain ER retention signals (KKXX or KKKXX), and tagged versions of Lag1p and Lac1p have been localized in yeast to the ER by immunofluorescence (Barz and Walter, 1999; P.A.Kirchman and S.M.Jazwinski, unpublished). This is the expected location of ceramide synthase, since mammalian ceramide synthase has been localized to the ER (Mandon *et al.*, 1992) and since other sphingolipid biosynthesis enzymes, such as keto-DSH reductase (Tsc10p) or DSH-hydroxylase (Sur2p), have ER retention motifs (Haak *et al.*, 1997; Beeler *et al.*, 1998) (Figure 1). ER retention signals are also present in *LAG1* homologues from *Schizosaccharomyces pombe*, man, mice, *Drosophila melanogaster* and *Caenorhabditis elegans*. (iii) The human homologue *LAG1Hs*, in spite of very limited homology to yeast *LAG1* and *LAC1*, can restore viability and a normal life span to yeast *lag1Δlac1Δ* mutants. Functional conservation is seen more often with enzymes than with regulatory subunits (Jiang *et al.*, 1998). (iv) As frequently found for housekeeping genes, the human *LAG1Hs* lacks a TATA box in the promoter (Jiang *et al.*, 1998). (v) The hydrophobic transmembrane domains of *LAG1* and its homologues can be predicted not only to have membrane-anchoring function, but also to carry out some specific function, since a large part of the conserved residues including the so-called Lag1p motif are found within the transmembrane domains (Jiang *et al.*, 1998; Barz and Walter, 1999).

Cells remaining viable in spite of the concomitant deletion of *LAG1* and *LAC1* show interesting adaptive phenomena. *lag1Δlac1Δ* mutants make novel mild base-resistant, inositol-containing lipids *in vivo* (Figure 2, lipids a and b), and they also generate huge amounts of an abnormal, [<sup>3</sup>H]DHS-labelled, base-resistant lipid, probably some kind of ceramide with a shorter chain FA (Figure 3, lipid α). Further structural studies will have to be undertaken in order to determine whether these lipids are related, i.e. whether lipids a and b contain lipid α. It is conceivable that the latter is a ceramide made by Ypc1p, a newly discovered alkaline ceramidase that can also catalyse the reverse reaction, namely the condensation of a FA with DHS or PHS (Mao *et al.*, 2000). Mao *et al.* (2000) show that microsomes from cells that overexpress Ypc1p make significantly increased amounts of labelled ceramide from [<sup>3</sup>H]palmitic acid and PHS, and they provide evidence suggesting that Ypc1p synthesizes ceramide in intact cells if they are treated with fumonisin B1. It is noteworthy that YBR183w (*YPCI*) was isolated as a multicopy suppressor of a *lag1Δlac1Δ* (P.A.Kirchman and S.M.Jazwinski, in preparation). In our microsomal assay, the synthesis of ceramide in the presence of C16 was, at best, very minor in comparison with the *LAG1/LAC1*-dependent synthesis of DHS-C26 (Figure 5C, lanes 1, 2 and 5). However, the conditions in our assays were quite different from those of Mao *et al.* (2000) since here Ypc1p was not overexpressed, incubations lasted only for 1 h, contained no detergent and no Ca<sup>2+</sup> but rather contained EGTA and ATP, and since the label was supplied as [<sup>3</sup>H]DHS. A compensatory mechanism also seems to provide some ceramide in the 2Δ<sup>sup</sup> strain, although for the moment we cannot tell what enzyme is synthesizing this lipid. In summary, our study lends support to the notion that yeast cells have alternative

ways of making ceramides, so that functional studies using inhibitors such as fumonisins or Aus have to be interpreted with great care.

A further interesting compensatory mechanism seems to be the up-regulation of the biosynthesis of long chain FAs and their incorporation into glycerophospholipids (Table I). It has been reported previously that *lcb1slc1-1* cells, being deficient in serine palmitoyltransferase, start to incorporate C26 FAs into PI when long chain bases are removed from their culture medium (Lester *et al.*, 1993; Nagiec *et al.*, 1993). Thus, these cells made a lipid that may be identical to PI' observed in [<sup>3</sup>H]inositol-labelled *lag1Δlac1Δ* cells (Figure 2, lanes 1–3). This previous study also showed that PI with a C26 FA in the *sn*-2 position of the glycerol moiety could serve as a substrate for the mannosyltransferase (MIPC synthase *CSG1*, *CSG2*; Zhao *et al.*, 1994; Beeler *et al.*, 1997) and M(IP)<sub>2</sub>C synthase (*IPT1*; Figure 1; Dickson *et al.*, 1997a), thus generating a class of lipids that resembles the natural sphingolipids with regard to the head groups as well as the presence of C26. Further studies into the exact nature of PI' and other abnormal lipids of *lag1Δlac1Δ* are under way. It is quite conceivable that the potential to induce compensatory mechanisms depends on the genetic background, thus explaining why the double deletion of *LAG1* and *LAC1* is lethal in some but not all genetic backgrounds.

The vesicular transport of GPI-anchored proteins out of the ER is singularly dependent on ongoing sphingolipid biosynthesis (Horvath *et al.*, 1994; Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997). The delay in transport of GPI-anchored proteins from ER to Golgi, which was observed in *lag1Δlac1Δ* cells (Barz and Walter, 1999), is likely to be secondary to the defect in ceramide biosynthesis in this mutant. This delay may result from inhibition or delay of GPI anchor attachment, a possibility raised by the 4-fold lower [<sup>3</sup>H]inositol incorporation into GPI-anchored proteins (Figure 8). Alternatively, and more probably, this low incorporation may be a reflection of a corresponding decrease in GPI-anchored protein biosynthesis in these slowly growing cells. Thus, it is not yet clear whether the apparent GPI protein transport deficiency of *lag1Δlac1Δ* cells is due to a problem with the anchoring or with the actual ER to Golgi transport of GPI-anchored proteins. Only if it is established that it is transport that is at fault can we interpret the data to mean that ceramide and not simply DHS, PHS, DHS-1-P or PHS-1-P is required for ER to Golgi transport of GPI-anchored proteins.

The identification of the role of *LAG1* and *LAC1* in sphingolipid metabolism provides a rationale for their impact on life span.

## Materials and methods

### Strains, growth conditions and materials

Strains used in this study are listed in Table III; the plasmids were described previously (Jiang *et al.*, 1998). Yeast cells were grown in YPD, SD or SDC medium (Benghezal *et al.*, 1996), supplemented as necessary, with the exception of W303-1A *lag1Δlac1Δ*, which was grown on SDCUA supplemented with 1 M sorbitol and 10 times the normal concentration of vitamins. Photometric determination of cell density was performed as described (Benghezal *et al.*, 1996), 1 OD<sub>600</sub> of cells corresponding to 1–2 × 10<sup>7</sup> cells. [<sup>3</sup>H]DHS was synthesized by NEN (Du Pont De Nemours, Les Ulis, France) and purified as described



**Table III.** Strains of *S.cerevisiae* used in this study

Strain	Genotype	Source
W303-1A	<i>MATa can1-100 ade2-1 his3-11,15 leu2-3 112 trp1-1 ura3-1</i>	S.M.Jazwinski
YPK9	<i>MATa ade2-101<sup>ochre</sup> his3-Δ200 leu2-Δ1 lys2-801<sup>amber</sup> trp1-Δ63 ura3-52</i>	S.M.Jazwinski
W3030-1A lac1Δ	same as W303-1A, but <i>lac1Δ::LEU2</i>	S.M.Jazwinski
W303-1A lag1Δ	same as W303-1A, but <i>lag1Δ::TRP1</i>	S.M.Jazwinski
W303-1A lac1Δlag1Δ	same as W303-1A, but <i>lac1Δ::LEU2 lag1Δ::TRP1</i>	S.M.Jazwinski
YPK9 lac1Δ	same as YPK9, but <i>lac1Δ::LEU2</i>	S.M. Jazwinski
YPK9 lag1Δ	same as YPK9, but <i>lag1Δ::TRP1</i>	S.M.Jazwinski
YPK9 lac1Δlag1Δ pBM150:LAG1	same as YPK9, but <i>lac1Δ::LEU2 lag1Δ::TRP1</i> containing pBM150:LAG1	S.M.Jazwinski
YPK9 lac1Δlag1Δ pBM150:LAC1	same as YPK9, but <i>lac1Δ::LEU2 lag1Δ::TRP1</i> containing pBM150:LAC1	S.M.Jazwinski
YPK9 pBM150:LAG1	same as YPK9, but containing pBM150:LAG1	S.M.Jazwinski
YPK9 pBM150:LAC1	same as YPK9, but containing pBM150:LAC1	S.M.Jazwinski
YPK9 pBM150	same as YPK9, but containing empty pBM150	S.M.Jazwinski
ypc1Δ	<i>MATa ura3-52 HIS3 leu2-3,112 TRP1 YBR183w(4948)::kanMX4</i>	EUROSCARF
acc1 ts	<i>MATa acc1-2150</i>	R.Schneiter
elo3Δ	<i>MATa ura3 trp1 leu2 elo3::URA3</i>	G.Loison and R.Schneiter
scs7Δ	<i>MATa trp1 leu2 his4 scs7Δ::URA3</i>	T.Dunn
sur2Δ	<i>MATa ura3-52 leu2 his4 sur2Δ::TRP1</i>	T.Dunn

(Reggiori *et al.*, 1997). AbA was from Takara Shuzo Co. (Shiga, Japan) and fumonisin B1 from Alexis Biochemicals (Läufelfingen, Switzerland). Aus was a generous gift of Suzanne Mandala (Merck, Rahway, NJ) and myriocin was a kind gift of Dr N.Rao Movva (Novartis, Basel, Switzerland).

#### Cell labelling, lipid extraction, treatment and thin-layer chromatography

Aus, AbA, fumonisin B1, myriocin and cycloheximide were added at concentrations of 2, 1, 10, 40 and 200 µg/ml, respectively. Unless stated otherwise, labelling, cell disruption, lipid extraction and analysis were carried out as described in Reggiori *et al.* (1997). Where indicated, lipids were subjected to mild base treatment using monomethylamine (MMA). For this, lipids were dissolved in MMA:water (10:3) and incubated for 1 h at 53°C. The samples were allowed to cool before being evaporated under vacuum in a Speed Vac evaporator. Strong HCl hydrolysis was carried out at 80°C in 2 M HCl for 16 h. Solvents 1, 2 or 3 were used for ascending TLC, namely 1 = chloroform:methanol:NH<sub>4</sub>OH (40:10:1), 2 = chloroform:methanol:0.25%KCl (55:45:10) and 3 = chloroform:methanol:0.25%KCl (55:45:5). Radioactivity was detected and quantitated by one-dimensional radioscanning and fluorography. Lipids to be analysed by GC-MS were always dried under a nitrogen stream instead of being vacuum evaporated, and were kept under nitrogen until injection. Labelling of cells with [<sup>3</sup>H]inositol for purification and analysis of GPI-anchored peptides was carried out as described (Guillas *et al.*, 2000).

#### GC-MS analysis of fatty acids

Dry lipids obtained from 100 OD of exponentially growing cells were incubated, either directly or after strong acid hydrolysis (see above), with 1 ml of 14% BF<sub>3</sub> in methanol for 2 min at 95°C. After methylation, 1 ml of water was added, and the methanol-water phase was extracted three times with 3 ml of petroleum ether. Pooled petroleum ether phases were washed with 2 ml of water before being dried under nitrogen. For injection, samples were resuspended in hexane:anhydrous ethyl ether (1:1). GC-MS analysis was performed as described (Oh *et al.*, 1997), using a 30 m × 0.25 mm Optima 05ms column with a film thickness of 0.25 µm. Gas chromatography data and mass spectra were analysed using the Xcalibur™ Finnigan corporation software.

#### Quantitation of long chain bases, free ceramides and total sphingolipids

Lipids were extracted as described in Nagiec *et al.* (1997) with 5 ml of chloroform:methanol (1:1) at 50°C. Lipids from 100 OD<sub>600</sub> were dried under a stream of nitrogen and were derivatized directly with the *o*-phthalaldehyde (OPA) reagent (Merrill *et al.*, 1988) to quantify free long chain bases. Alternatively, to isolate free ceramides, dried lipids were resuspended in 0.5 ml of chloroform and applied to a 1 ml silica gel 100–200 mesh (Sigma, S4133) column equilibrated in chloroform. After washing with 3 ml of chloroform, the free ceramides were eluted with 3 ml

of chloroform:methanol (9:1) (Nagiec *et al.*, 1997). The eluted ceramides were dried and subjected to strong acid hydrolysis before OPA derivatization. Derivatized PHS and DHS were separated and quantified by HPLC on a 25 × 0.46 Nucleosil 100-5 C18 column, eluted with methanol–5 mM K<sub>2</sub>HPO<sub>4</sub> pH 7.0 (90:10) at a flow rate of 0.8 ml/min and monitored at 340 nm.

#### Synthesis of C26-CoA

The protocol was adapted from Hajra and Bishop (1986) for non-radioactive FAs. Briefly, 30 µmol of C26 were dried thoroughly three times with 1 ml of dry benzene under a gentle stream of nitrogen. A 1.6 ml aliquot of dry benzene and 0.8 ml of oxalyl chloride were added to the FA residue and incubated for 90 min under nitrogen at room temperature. Liquids were evaporated with dry nitrogen and the oxalyl chloride residue was dried three times with dry benzene under dry nitrogen. A 35 µmol concentration of CoA lithium salt dissolved in 2.6 ml of tetrahydrofuran–aqueous 150 mM NaHCO<sub>3</sub> (2.2:1.0) (adjusted to pH 8.8 with NaOH) was added to the acyl chloride and incubated for 2 h at 37°C under nitrogen with vigorous stirring. The reaction was stopped by adding 40 µl of 10% HClO<sub>4</sub>. The tetrahydrofuran was evaporated under dry nitrogen. The residue was then washed by repeated resuspension and centrifugation (18 000 g, 15 min at 4°C): twice with 5 ml of 1.3% HClO<sub>4</sub>, once with 8 ml of acetone, twice with 8 ml of anhydrous ether and finally with 8 ml of tetrahydrofuran. The residue was dried under nitrogen and resuspended in 0.5 mM Zwittergent 3-16. The concentration of C26-CoA was then evaluated by its absorbance at 260 nm (ε = 16 800).

#### Labelling of microsomes with [<sup>3</sup>H]DHS

Microsomes from 2.5 OD of cells were prepared as described (Canivenc-Gansel *et al.*, 1998). They were labelled with 50 µCi of [<sup>3</sup>H]DHS for 1 h at 37°C in labelling buffer containing 100 mM Tris–HCl pH 7.5, 1 mM EGTA, 3 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, 1 mM ATP, 30 mM creatine phosphate and 1 mg/ml creatine kinase. CoA, C26-CoA, C16-CoA, C26 and C16 were added to achieve final concentrations of 1.0, 0.1, 0.14, 0.1 and 0.1 mM, respectively, only where indicated.

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