A novel pathway of ceramide metabolism in *Saccharomyces cerevisiae*

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The hydrolysis of ceramides in yeast is catalysed by the alkaline ceramidases Ypc1p and Ydc1p, two highly homologous membrane proteins localized to the ER (endoplasmic reticulum). As observed with many enzymes, Ypc1p can also catalyse the reverse reaction, i.e. condense a non-esterified fatty acid with PHS (phytosphingosine) or DHS (dihydrosphingosine) and thus synthesize ceramides. When incubating microsomes with [³H]palmitate and PHS, we not only obtained the ceramide PHS–[³H]$C_{16:0}$, but also a more hydrophobic compound, which was transformed into PHS–[³H]$C_{16:0}$ upon mild base treatment. The biosynthesis of a lipid with similar characteristics could also be observed in living cells labelled with [¹⁴C]serine. Its biosynthesis was dependent on the diacylglycerol acyltransfereases Lro1p and Dga1p, suggesting that it consists of an acylceramide. The synthesis of acylceramide could also be monitored using fluorescent NBD (7-nitrobenz-2-oxa-1,3-diazole)–ceramides as an acceptor substrate for microsomal assays. The Lro1p-dependent transfer of oleic acid on to NBD–ceramide was confirmed by high-resolution Fourier transform and tandem MS. Immunopurified Lro1p was equally able to acylate NBD–ceramide. Lro1p acylates NBD–ceramide by attaching a fatty acid to the hydroxy group on the first carbon atom of the long-chain base. Acylceramides are mobilized when cells are diluted into fresh medium in the presence of cerulenin, an inhibitor of fatty acid biosynthesis.

Key words: acylceramide, acyltransferase, detoxification, DGA1, LRO1, triacylglycerol.

INTRODUCTION

Sphingolipids are essential structural components of cell membranes that also have messenger functions regulating the proliferation, survival and death of cells. The yeast sphingolipids consist of LCBs (long-chain bases), LCB-1-phosphates, ceramides, IPCs (inositolphosphorylceramides) and their mannosylated derivatives [1]. Sphingolipid synthesis begins in the ER (endoplasmic reticulum), where serine palmitoyltransferase uses serine and a fatty acyl-CoA to make 3-oxo-sphinganine, which is then converted into DHS (dihydrosphingosine; sphinganine). DHS is then hydroxylated at C4 by Sur2p yielding its 4-hydroxy derivative PHS (phytosphingosine; 4-hydroxysphinganine). Ceramides are made in the ER, whereas the biosynthesis of IPCs and more complex sphingolipids occurs in the Golgi [2]. Thus ceramide is an intermediate in the formation of complex sphingolipids. In mammalian cells, many reports have documented the important role of ceramides as signalling molecules [3].

In the yeast *Saccharomyces cerevisiae* the biosynthesis of ceramide is mainly achieved by the acyl-CoA-dependent ceramide synthase *LAG1* and its close homologue *LAC1*, formerly known as longevity assurance genes [4,5]. Ceramides then can follow any one of several pathways, as indicated in Figure 1. Ceramides can also be hydrolysed by Ypc1p and Ydc1p, two alkaline ceramidases that display more then 50% of homology over their entire amino acid sequence and reside in the ER, where Lag1p and Lac1p are also localized. Ypc1p catalyses, *in vitro* and in certain instances *in vivo*, the reverse reaction, i.e. the condensation of non-esterified fatty acids with PHS or DHS [6]. Ydc1p, in contrast with Ypc1p, has been shown to hydrolyse only DHS-containing ceramides and exhibits only very weak reverse activity *in vitro*, but seems to be able to work in the reverse direction *in vivo* [5,7–9].

Like many organisms, yeast store neutral lipids in the form of LDs (lipid droplets). Synthesis of neutral lipids and thus LD biogenesis in yeast is mediated by two acyl-CoA:sterol acyltransferases, Are1p and Are2p, and by Lro1p and Dga1p, which acylate DAG (diacylglycerol) to produce TAG (triacylglycerol). All of these enzymes are present in the ER, except for Dga1p which has a much higher specific activity in LDs than the ER [10–12]. *LRO1* accounts for the major part of TAG biosynthesis in exponentially growing cells and is homologous with the mammalian LCAT (lecithin cholesterol acyltransferase) [13,14]. Lro1p transfers a fatty acid from the *sn*-2 position of PE (phosphatidylethanolamine) or PC (phosphatidylcholine) on to DAG, whereas Dga1p transfers the fatty acid from acyl-CoA on to DAG [10,13–15]. Neutral lipid synthesis and storage are dispensable for the viability of *S. cerevisiae* because an *are1Δare2Δdga1Δlro1Δ* quadruple mutant is viable, makes no storage lipids and lacks detectable LDs [14,16].

In the present paper we report on the enzymatically mediated acylation of ceramides, a process which physiologically may allow for ceramide storage or detoxification.

EXPERIMENTAL

### Strains, growth conditions and materials

S. cerevisiae strains used are listed in Table 1 and plasmids used are listed in Table 2. Mutant strains were generated using standard methods for crossing of single mutants, for plasmid transfection or gene disruption using deletion cassettes generated

Table 1   Yeast *S. cerevisiae* strains

Strains	Genotype	Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCARF
WT.vector (FBY5218)	As BY4742 but also containing pRS415	The present study
WT.YPC1 (FBY5215)	As BY4742 but also containing pYPC1-LEU2	The present study
lro1Δ.YPC1 (FBY5219)	As BY4742, but *lro1::kanMX* and also containing pYPC1-LEU2	The present study
lro1Δ.YPC1.LRO1 (FBY5277)	As BY4742, but *lro1::kanMX* and also containing pYPC1-URA3 and pLRO1-LEU2	The present study
ypc1Δydc1Δ.vector (FBY5322)	*MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ypc1::LEU2 ydc1::natMX* containing pNP302	The present study
ypc1Δydc1Δ.YPC1 (FBY5321)	*MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ypc1::LEU2 ydc1::natMX* containing pYPC1-URA3	The present study
are1Δare2Δlro1Δ.DGA1 (RSY3292)	As BY4742 but *are1::kanMX are2::kanMX trp1::URA3 lro1::loxP GAL1_{UAS}-GFP-DGA1::HIS3*	[12]
are1Δare2Δdga1Δ.LRO1 (RSY3202)	As BY4742 but *are1::kanMX are2::kanMX trp1::URA3 dga1::loxP GAL1_{UAS}-GFP-LRO1::HIS3*	R. Schneiter
are1Δlro1Δdga1Δ.ARE2 (RSY3492)	As BY4742 but *lro1::kanMX dga1::loxP are1::loxP GAL1_{UAS}-ARE2::HIS3*	R. Schneiter
scs7Δ	BY4742, but *scs7::kanMX*	EUROSCARF
isc1Δ	BY4742, but *isc1::kanMX*	EUROSCARF
are1Δare2Δdga1Δ.LRO1.DGA1 (RSY4360)	As BY4742 but *are1::kanMX are2::kanMX trp1::URA3 dga1::loxP GAL1_{UAS}-LRO1::TRP1* containing pGAL1-GFP-DGA1-URA3	R. Schneiter
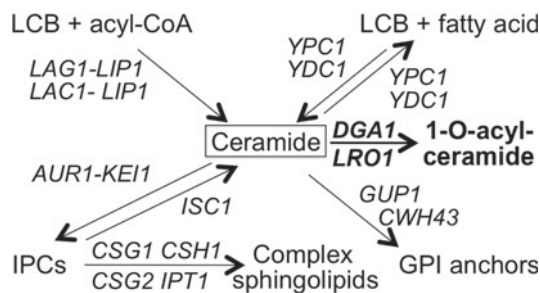
Figure 1   Ceramide biosynthesis and utilization in yeast

The various metabolic pathways generating and consuming ceramides are shown. Gene names are in italic. The novel pathway generating acylceramides described in the present paper is in bold.

Table 2   Plasmids

Plasmid	Details	Reference
pRS415	CEN ARS *LEU2*	[45]
pNP302	CEN ARS *URA3, ADH1* promoter	C. De Virgilio
pNP308	CEN ARS *LEU2, ADH1* promoter	C. De Virgilio
pYPC1-LEU2	*YPC1* in pNP308	The present study
pYPC1-URA3	*YPC1* in pNP302	The present study
pLRO1-LEU2	*LRO1* behind its natural promoter in pRS415	R. Schneiter

by PCR. Cells were grown on rich medium {YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or YPG [1 % (w/v) yeast extract/2 % (w/v) tryptone/ 2 % (w/v) galactose]}, or synthetic minimal or complete media [YNB (yeast nitrogen base); United States Biological) containing 2 % (w/v) glucose (D) or galactose (G) as a carbon source. All experiments were performed with cells growing exponentially. Where indicated, medium or buffers were supplemented with CHX (cycloheximide), myriocin, cerulenin, palmitate or L-α-PC from egg yolk, all obtained from Sigma. [14C]Serine and [3H]palmitate were from American Radiolabeled Chemicals. DHS, PHS, fluorescent ceramides (C₁₂–NBD–PHS and C₆–NBD–DHS; NBD is 7-nitrobenz-2-oxa-1,3-diazole) and fatty acyl-CoAs were from Avanti Polar Lipids. C₆–NBD–sphingosine was from Invitrogen. Monomethylamine (33 % in ethanol) was from Fluka. Dynabeads®–Protein G was from Invitrogen. Zymolyase-20T (*Arthrobacter luteus*) was from Seikagaku Biobusiness. Mouse monoclonal anti-GFP (green

fluorescent protein) antibodies were from Roche Diagnostics. The anti-mouse IgG–peroxidase conjugate was from Sigma.

Construction of plasmids

To construct plasmids containing *YPC1*, the open reading frame was amplified by PCR using the oligonucleotides 5′-CCT-GGGATCCATGGGAATATTTCGTTGGAACTATCC-3′ and 5′-CGCCGCGGCCGCTTACTTCTCCTTTTTAACTTC-3′ and genomic DNA from WT (wild-type) BY4742 cells. PCR products were doubly digested with NotI and BamHI and ligated into the similarly digested pNP308 or pNP302 vectors to generate pYPC1-LEU2 and pYPC1-URA3. DNA sequencing of inserts confirmed the correct sequence of *YPC1*.

Protein analysis

Proteins were extracted by mild alkali treatment and subsequent boiling of cells in a standard electrophoresis loading buffer [17]. Proteins were resolved by SDS/PAGE and detected by Western blotting using standard procedures.

Mass spectrometric lipid analysis

Microsomes were extracted with chloroform/methanol [2:1 (v/v)] [18]. Lipid extracts were analysed in negative-ion mode by direct infusion MS using an LTQ Orbitrap XL mass spectrometer equipped with the automated nanoflow ion source Triversa NanoMate (Advion Biosciences) [18]. C₆–NBD–DHS and its acyl-derivatives were detected by high-resolution FT-MS (Fourier transform MS) using a target mass resolution of 100 000. In addition, the identity of detected C₆–NBD–DHS and acyl-derivatives was confirmed by ion-trap MS/MS (tandem MS) analysis.

Metabolic labelling of cells with [14C]serine, lipid extraction, mild base treatment and TLC

Cells were grown in synthetic minimal medium. Then 3.0 D_{600} units of exponentially growing cells (3 ml of a culture having a D_{600} of 1.0) were harvested and resuspended in 250 μl of the same medium supplemented with 10 μg/ml CHX. After 10 min of pre-incubation, 4 μCi of [14C]serine was added and cells were incubated for 40 min at 30°C. Then the samples were diluted with 750 μl of fresh minimal medium supplemented with CHX and labelling was continued for a further 120 min. Labelling

was terminated by adding sodium azide and sodium fluoride (10 mM final concentrations) and chilling cells on ice. Cells were resuspended in chloroform/methanol [2:1 (v/v)] and broken with glass beads in the cold. The extract was kept apart and the pellet was re-extracted sequentially with chloroform/methanol [1:1 (v/v)] and ethanol/water/diethyl ether/putridine/25 % ammonium hydroxide (15:15:5:1:0.018, by vol.), which achieves quantitative extraction of all complex sphingolipids [19]. Extracts were combined and solvent was evaporated under vacuum in a rotary evaporator. Incorporation into lipids usually amounted to 5 % of radioactivity added. Where indicated, lipids were subjected to mild base hydrolysis with sodium hydroxide or MMA (monomethylamine). For this, lipids were resuspended in 200 μl of chloroform/methanol/water (10:10:3, by vol.), 40 μl of 0.6 M sodium hydroxide in methanol (final concentration of 0.1 M) was added, and samples were incubated for 1 h at 37 °C. Hydrolysis was stopped with 40 μl of 0.8 M acetic acid in methanol. Control samples were incubated on ice and at the end of the incubation supplemented with 40 μl of 0.8 M acetic acid plus 40 μl of 0.6 M sodium hydroxide. The desalted lipids were resolved by ascending TLC on silica gel plates. Alternatively, lipids were resuspended in 400 μl of MMA (33 % in ethanol) or, as a negative control, in methanol, and incubated at 53 °C for 1 h. Then, solvents were evaporated under vacuum. All lipids were resolved by ascending TLC on silica gel plates after having been desalted by Folch partitioning as described previously [20]. Unless indicated otherwise, extracts of microsomes were developed with solvent 1 (chloroform/methanol/25 % ammonium hydroxide, 9:2:0.5, by vol.) and extracts from metabolically labelled cells were developed with solvent 2 (chloroform/methanol/2 M ammonium hydroxide, 40:10:1, by vol.). When the untreated and deacylated lipid extract was run side by side, material from an equivalent number of cells was spotted, whether we analysed extracts from metabolic labellings or microsomal labelling reactions (see below). Radioactivity was detected and quantified by one- and two-dimensional radioscanning using a Berthold radioscanner and visualized by fluorography or radioimaging using the Bio-Rad Molecular Imager FX. Unless otherwise stated, radioactivity in individual spots was given as the percentage of total radioactivity detected by radioscanning in the corresponding lane, except for acylceramides. In the case of acylceramides, the counts of residual mild-base-resistant lipid co-migrating with acylceramides, as well as of an occasional trace of material co-migrating with fatty acids, were deducted from the acylceramide signal as follows:

Percentage of acylceramides

= (acylceramide untreated − [acylceramide deacylated

+ fatty acid deacylated]/total counts untreated) × 100.

Preparation of microsomes

Microsomes were prepared from yeast cells grown in synthetic complete media. Briefly, as described previously [6], cells were homogenized in a lysis buffer containing 25 mM Tris/HCl (pH 7.4), 1 mM PMSF and Roche protease inhibitor cocktail. Unbroken cells and cell debris were removed by centrifugation at 500 g for 5 min. The membrane fraction was sedimented by centrifuging the supernatant at 16 000 g for 40 min at 4 °C. The microsomes were resuspended in assay buffer A containing 25 mM Tris/HCl (pH 8.0), 5 mM CaCl$_2$ and 1 mM PMSF. In most experiments, boiled microsomes (100 °C for 10 min) were used as a negative control. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories).

Microsomal assay of reverse ceramidase using [³H]palmitate

The reverse ceramidase activity was measured as described previously [21] with the following modifications: 10 μl of 1 mM PHS or DHS, 3 μCi of [³H]palmitate (60 Ci/mmol) and 0.4 nmol of non-radioactive palmitate were dried under vacuum in a rotary evaporator. Dried lipids were dissolved in 20 μl of buffer A by water bath sonication. The reactions were started by adding microsomes corresponding to 50 μg of protein to attain a final volume of 50 μl. Samples were incubated at 30 °C for 1 h at 300 rev./min on a tube shaker. The reactions were terminated by adding 300 μl of chloroform/methanol [2:1 (v/v)].

Microsomal assays using NBD–ceramides

NBD-containing ceramides (C$_{12}$–NBD–PHS, C$_6$–NBD–DHS or C$_6$–NBD–sphingosine) were bound to BSA using an established procedure [22], which was modified as follows. For ten standard reactions, 100 nmol of NBD–ceramide were dissolved in 20 μl of ethanol and added to 200 μl of buffer A containing 40 mg/ml of fatty-acid-free BSA giving an NBD–ceramide to BSA molar ratio of 1:1. The solution was incubated on a wheel at 4 °C for 2 h. Microsomal pellets were dissolved in buffer A supplemented with 10 mg/ml of fatty-acid-free BSA, the final concentration of microsomal protein being 5.0 mg/ml. The reactions were initiated by adding 50–100 μg of microsomes to 10 nmol of NBD–ceramide in a final volume of 50 μl containing 10 mg/ml BSA. Reaction mixtures were incubated at 30 °C for 2 h and then stopped by adding 300 μl of chloroform/methanol [2:1 (v/v)]. Lipids were extracted, treated or not with mild base, desalted and resolved by TLC. Products of NBD–ceramide conversion were identified as fluorescent bands and quantified with a FluorChem 8900 fluorescence detector (Witec).

## Immunopurification of Lro1p–GFP on Dynabeads®–Protein G

RSY3202 cells were grown on YPG media. Cells were collected, treated with zymolyase and spheroplasts were lysed with a Dounce homogenizer in buffer B [20 mM Hepes (pH 6.8), 0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA and protease inhibitors]. Microsomes were sedimented by centrifugation at 16 000 g for 30 min at 4 °C and solubilized in buffer B with 0.5 % Triton X-100 for 30 min on ice whereupon non-solubilized material was removed by centrifugation. Next, 4 μg of mouse anti-GFP antibodies were bound to 50 μl (1.5 mg) of Dynabeads and the washed beads were incubated with 500 μg of solubilized microsomal protein for 30 min at room temperature (25 °C) on a rotating wheel. Beads were sedimented using a magnet, the supernatant containing non-bound proteins was removed and the beads were washed three times with 0.5 % Triton X-100 in PBS using magnetic sedimentation. Acylceramide synthesis was measured in a final volume of 90 μl for 2 h at room temperature. The assay contained buffer A, BSA (10 mg/ml), PC (450 μM), C$_6$–NBD–DHS (50 μM), a final concentration of 0.5 % Triton X-100 and 20 μl of Dynabeads–Lro1p–GFP conjugate or supernatant containing non-bound proteins. For protein determination, purified Lro1p–GFP was eluted from beads at pH 2.5.

RESULTS

In vitro microsomal assay of ceramide synthase activity reveals Lro1p-dependent ceramide esterification

As described previously [6], reverse ceramidase activity of Ypc1p can be measured by incubating microsomes with [³H]palmitate and non-radioactive LCBs (Figures 2A and 2B). The R_f of
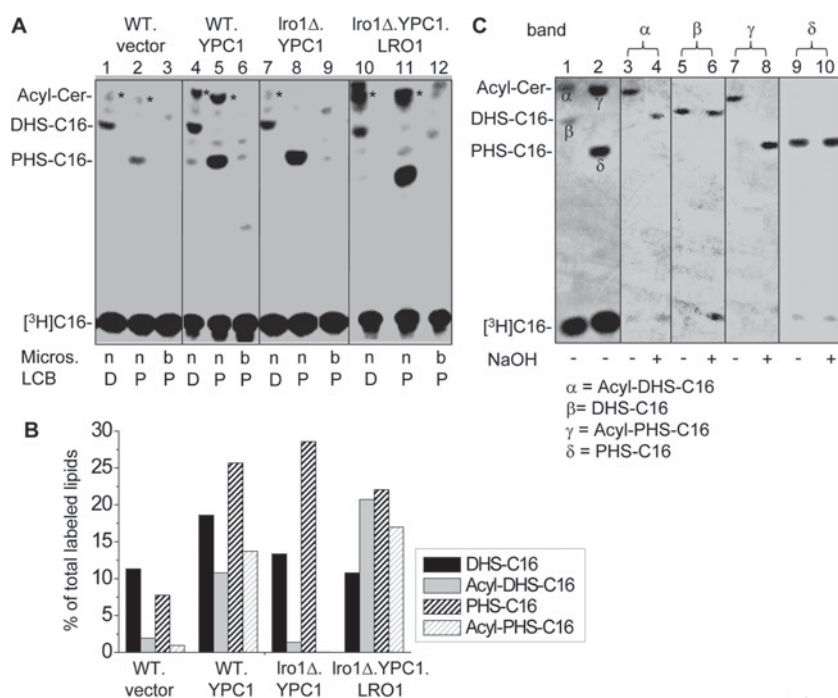
Figure 2   Lro1p-dependent ceramide esterification in microsomes

(**A**) Microsomes were prepared from BY4742 (WT) and *lro1Δ* cells carrying an empty vector or vectors with open reading frames of the genes indicated after the dots. The reverse activity of Ypc1p was measured by incubating native (n) or boiled (b) microsomes with [³H]palmitate and DHS (D) or PHS (P) as substrates. Lipids were extracted, resolved by TLC and quantified by radioscanning. Presumed acylceramides are marked with an asterisk. A representative experiment from three experiments, all giving the same result, is shown. (**B**) Counts present in ceramides and putative acylceramides of (**A**) were quantified by radioscanning and plotted as a percentage of the total counts present in the lane. (**C**) Presumed acylceramides and ceramides obtained in an *in vitro* assay with microsomes from WT vector cells using DHS (lane 1) or PHS (lane 2) as substrates were labelled α, β, γ and δ. These bands were scraped off of the TLC plate, extracted and treated with mild base (sodium hydroxide) or incubated as a control. Hydrolysis products were desalted and separated by TLC. The identity of lipids α–δ on the basis of this experiment is indicated at the bottom.

ceramides is higher when DHS is used as a substrate than when PHS is used (Figure 2A) and the reaction is much faster when *YPC1* is overexpressed (Figure 2A, lanes 1 and 2 compared with lanes 4 and 5, and Figure 2B). (Microsomes from Ypc1p-overexpressing cells actually represent an easy and cheap way to generate radiolabelled ceramides.) The reverse ceramidase assay invariably also generated a lipid, which migrated faster than ceramides, pointing to its higher hydrophobicity as compared with ceramides (marked with an asterisk in Figure 2A). We will show below that this species is likely to be a 1-O-acylated ceramide. O-acylated ceramides, as well as ceramides, did not appear when boiled microsomes were used (Figure 2A, lanes 3, 6, 9 and 12). The putative acylceramides were scraped off the TLC plate and treated with sodium hydroxide for saponification (Figure 2C). Upon mild base hydrolysis the presumed acylceramides α and γ, generated using either DHS or PHS as substrates, yielded the corresponding ceramides (lanes 3, 4, 7 and 8); the ceramides themselves (β and δ) remained stable in the presence of sodium hydroxide (lanes 5, 6, 9 and 10), in agreement with the idea that the lipids marked with an asterisk in Figure 2(A) are acylceramides.

Acylceramide had previously been shown to be made by mammalian cells when microsomes were incubated with *N*-acetyl-[³H]sphingosine. The enzyme catalysing the synthesis of *O*-acylceramide in mammals was identified as LPLA2, a soluble lysosomal enzyme working mainly as an acid PLA₂ (phospholipase A₂), but able to also use ceramide rather than water as an acceptor substrate [23]. A BLAST search showed that its closest homologue in *S. cerevisiae* is Lro1p. Indeed, the synthesis of acylceramides was strongly reduced in *lro1Δ*

cells, suggesting that *LRO1* deletion abolished ceramide acylation (Figure 2A, lanes 7 and 8 compared with lanes 4 and 5). Moreover, the complementation of the *lro1Δ* mutant with a single copy plasmid bearing *LRO1* under its native promoter completely rescued the acylation of ceramides (Figure 2A, lanes 10 and 11). We wondered whether the second yeast DAG acyltransferase, Dga1p, is also capable of catalysing the synthesis of acylceramide *in vitro*. To test for this we incubated microsomes of an *lro1Δ* strain overexpressing *YPC1* with PHS and [³H]palmitate as in Figure 2(A), lane 8, in order to allow for the formation of labelled ceramides. At the end of the incubation, the reaction was supplemented with different acyl-CoAs as acyl donors for Dga1p and the incubation was continued for a further 1 h (Supplementary Figure S1 at http://www.BiochemJ.org/bj/447/bj4470103add.htm). No acylceramide synthesis was detected in this experiment. Although Dga1p is able to acylate ceramides *in vivo* (see below), reasons for this negative *in vitro* experiment could be that Ypc1p and Dga1p reside in different subcompartments of the ER, which end up in different microsomes, i.e. that microsomes generating [³H]ceramide did not contain enough Dga1p activity. Indeed, Dga1p activity is concentrated in LDs, whereas Lro1p is mainly present in the ER membrane [10,13] (R. Schneiter, personal communication).

NBD–ceramides are acylated *in vitro* by Lro1p

To further support the idea that Lro1p is capable of O-acylating ceramides, we tested the capacity of microsomes to acylate fluorescent ceramides. Figure 3(A) demonstrates that up to 25 % of C₁₂–NBD–PHS was acylated by microsomes in a 2 h assay,
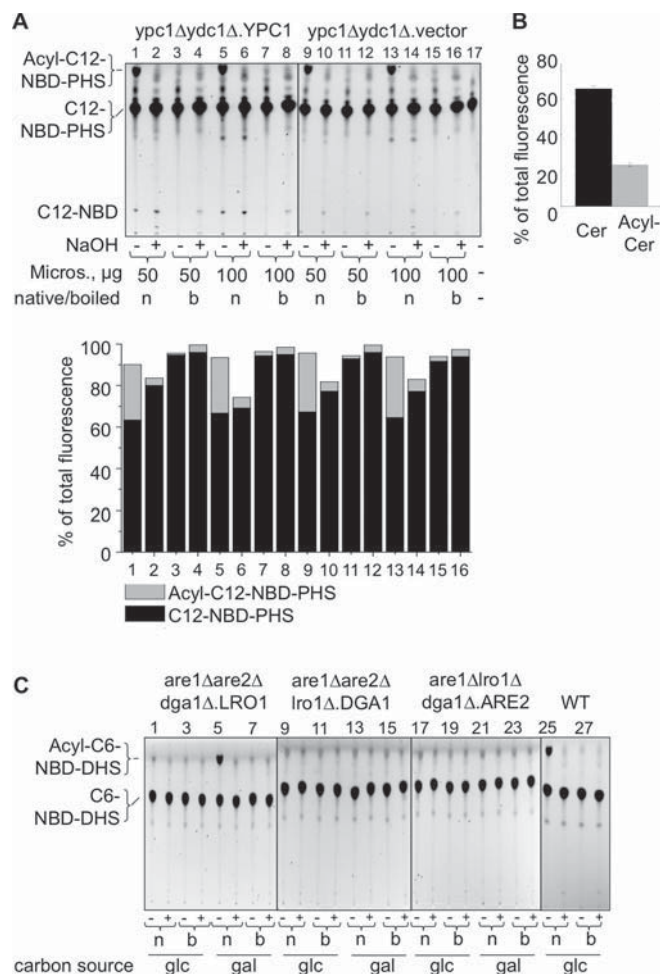
Figure 3 *In vitro* acylation of NBD–ceramides

(**A**) Native or boiled microsomes from the indicated strains were incubated for 2 h at 30 °C in the presence of C_{12}–NBD–PHS, i.e. a ceramide containing an NBD–C_{12} fatty acid linked to PHS. Lipids were extracted, subjected or not to alkaline hydrolysis and resolved by TLC. Lane 17 contains the C_{12}–NBD–PHS standard as received from Avanti Polar Lipids. The bottom panel represents the quantification of acylceramide and ceramide as a percentage of total fluorescence in each lane. (**B**) Results from (**A**) were quantified by averaging fluorescent signals of ceramides and acylceramides of lanes 1, 5, 9 and 13. (**C**) The indicated mutants were grown on glucose or galactose overnight. Microsomes were labelled as described above, but using C_6–NBD–DHS, i.e. an NBD-C_6 fatty acid linked to the nitrogen of DHS. Microsomes of are1Δare2Δlro1Δ.DGA1 and are1Δlro1Δdga1Δ.ARE2 strains were additionally supplemented with 5 nmol of $C_{16:0}$-CoA and $C_{18:1}$-CoA respectively. b, boiled; Cer, ceramide; glc, glucose; gal, galactose; n, native.

and the extent of this acylation was independent of the presence of Ypc1p in the *ypc1Δydc1Δ* background used in the present experiment (Figures 3A and 3B). However, when Ypc1p was present, we observed a faint signal, presumably corresponding to C_{12}–NBD fatty acid, the product of the ceramidase activity of Ypc1p (Figure 3A, lanes 1 and 5). Qualitatively similar results were obtained with C_6–NBD–DHS, having a shorter NBD–fatty acid and a different LCB (Figure 3C, lanes 5 and 25, and Figure 4D), whereby no ceramidase activity was detected, possibly because *YPC1* was not overexpressed as in Figure 3(A). Acylation, as well as hydrolysis of NBD–ceramides, were observed to a much lower degree when boiled microsomes were used in the reaction (Figure 3A, lanes 3, 7, 11 and 15 compared with lanes 1, 5, 9 and 13, and Figure 3C). The rate of C_{12}–NBD–PHS hydrolysis by Ypc1p-containing microsomes in Figure 3(A) amounted to approximately 1 %

of total fluorescence (lanes 1 and 5), i.e. it was 6-fold faster than the hydrolysis by yeast microsomes strongly overexpressing human alkaline ceramidase [21]. Although we did not study the kinetics of the metabolism of NBD–ceramides in microsomes, it nevertheless is striking to observe that acylation of this artificial substrate by endogenous levels of Lro1p is 20-fold faster than its hydrolysis by the overexpressed Ypc1p (Figure 3A, lanes 1 and 5), whereby the conditions used for acylceramide synthesis in Figure 3(A) are the same as the ones optimized for measuring Ypc1p-mediated ceramidase activity [6]. Mild base treatment also released approximately 1 % of the amide-bound NBD–C_{12} fatty acids (Figure 3A, lanes 4, 8, 12 and 16 compared with lanes 3, 7, 11 and 15).

To see which enzymes were responsible for NBD–ceramide acylation, we took advantage of *are1Δare2Δlro1Δ.DGA1*, *are1Δare2Δdga1Δ.LRO1* and *are1Δlro1Δdga1Δ.ARE2* strains, in which the endogenous *DGA1*, *LRO1* or *ARE2* gene is placed behind the *GAL1*-promoter respectively, while all other DAG- or sterol-acyltransferases are deleted. These mutants were cultured on glucose or galactose to repress the expression or induce the overexpression of *LRO1*, *DGA1* or *ARE2* respectively. (Are2p accounts for most of the sterol acyltransferase activity, when yeast cells grow in the presence of oxygen [24].) Massive amounts of acylated C_6–NBD–DHS were made by microsomes derived from cells overexpressing Lro1p after having been grown on galactose, much more than from cells grown on glucose (Figure 3C, lanes 1 and 5). In contrast, overexpression of Are2p or of Dga1p did not result in significant microsomal ceramide acylation in the presence of acyl-CoA (Figure 3C).

Identification of the hydroxy group that is acylated by Lro1p

PHS has hydroxy groups on carbon atoms 1, 3 and 4, and DHS on carbon atoms 1 and 3. To decide whether ceramides are acylated on the carbon atom 1 or 3 of the LCB, we used a procedure previously employed by others [25], which requires the use of ceramides containing sphingosine rather than DHS or PHS. Sphingosine is a dehydrogenated DHS with a Δ4 double bond (Figure 4A). We found that yeast microsomes efficiently acylate C_6–NBD–sphingosine (Figure 4B). Acyl-C_6–NBD–sphingosine and unreacted C_6–NBD–sphingosine were scraped from a TLC plate and treated with DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) [25]. DDQ oxidizes α-β-unsaturated alcohols to ketones. Thus an hydroxy group in the C3 position of sphingosine will be oxidized to a ketone group, unless it is acylated (Figure 4A). After treatment with DDQ, all of the C_6–NBD–sphingosine was converted into corresponding 3-oxo-forms, which have a higher TLC mobility, as described previously [25] (Figure 4C, lanes 1 and 2). The same was true for acyl-C_6–NBD–sphingosine (Figure 4C, lanes 3 and 4). Controls showed that C_6–NBD–DHS and acyl-C_6–NBD–DHS lacking the Δ4 double bond were not oxidized (Figures 4D and 4E). These results suggest that the microsomal ceramide esterification by Lro1p involves the hydroxy group on C1, not C3, of the LCB.

Lro1p transfers oleic acid on to NBD–ceramide

If Lro1p is the enzyme that acylates ceramides, we would expect that it transfers on to ceramides the same C_{16} and C_{18} fatty acids as to DAG. To test this, microsomes from WT cells were incubated with the ceramide analogue C_6–NBD–DHS. Microsomes were subsequently extracted and analysed by high-resolution FT-MS and structural analysis by ion-trap MS/MS [18,26]. FT-MS analysis detected a molecular ion with *m/z* 840.6215 in microsomes incubated with
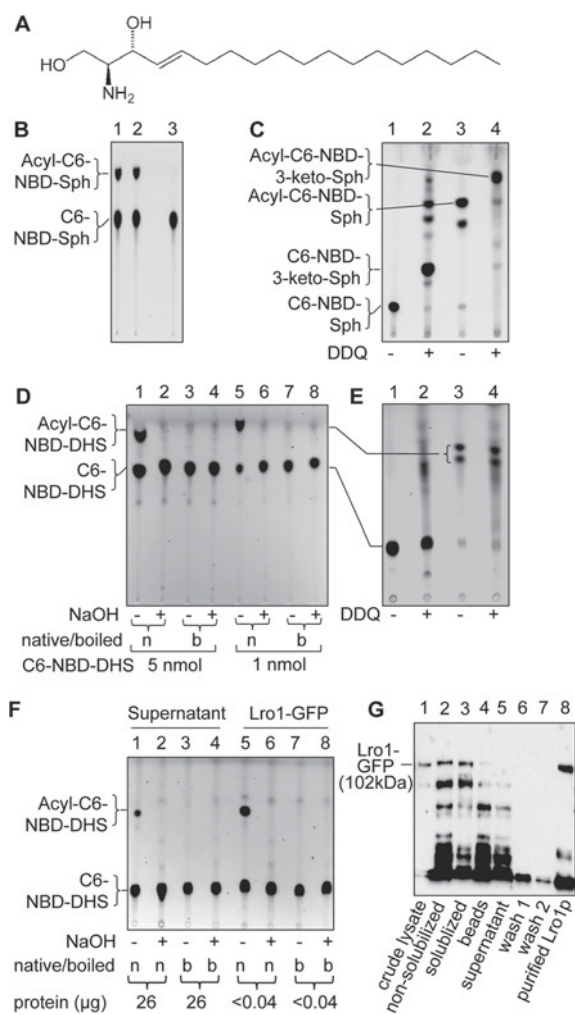
Figure 4   Lro1p acylates the hydroxy group at C1 of ceramides

(**A**) Formula of D-erythro-sphingosine. (**B**) The experiment shown in Figure 3(C), lane 5 was repeated using C_6–NBD–sphingosine as a substrate instead of C_6–NBD–DHS (lanes 1 and 2). Lane 3 contains the C_6–NBD–sphingosine as obtained from Invitrogen. Lipids were extracted and resolved by TLC. (**C**) Acyl-C_6–NBD–sphingosine and C_6–NBD–sphingosine from (**B**) were scraped off of the plate. Lipids were extracted from the silica and incubated for 48 h at 37 °C with or without 3 % DDQ in 40 μl of dioxane and then dried in a rotary evaporator as described previously [25]. The lipids were resuspended in 3 ml of chloroform/methanol [2:1 (v/v)] and 0.6 ml of 0.1 M sodium hydroxide was added. After vigorous shaking and brief centrifugation the lower layer (organic phase) was recovered and re-extracted twice with 2 ml of methanol/0.1 M sodium hydroxide [1:1 (v/v)] and twice more with 2 ml of methanol/water [1:1 (v/v)]. The lipids were resolved by TLC in chloroform/methanol/glacial acetic acid (90:1:9, by vol.) and then visualized by direct fluorescence using a FluorChem fluorescence detector. Oxidation of the C3 hydroxy group increases the mobility in this solvent system [25]. (**D**) The same experiment as in (**B**) was performed using 5 or 1 nmol of C_6–NBD–DHS as a substrate. (**E**) Acyl-C_6–NBD–DHS and C_6–NBD–DHS of (**D**) were scraped and processed as in (**C**). (**F**) Lro1p–GFP was purified using an anti-GFP antibody. Equivalent aliquots of purified Lro1p–GFP bound to affinity beads (lanes 5–8) or of supernatant containing non-bound microsomal proteins were incubated with C_6–NBD–DHS and PC for 2 h at room temperature. Lipids were extracted and separated on TLC as in (**C**). (**G**) Aliquots corresponding to equivalent amounts of starting material taken throughout purification were analysed by Western blotting using an anti-GFP antibody. b, boiled; n, native; Sph, sphingosine.

C_6–NBD–DHS, but not in microsomes without the addition of C_6–NBD–DHS or if microsomes had been boiled (Figures 5A–5D). With a mass accuracy of 0.6 p.p.m., this molecular ion matches the identity of the acylceramide $C_{18:1}$-C_6–NBD–DHS (calculated m/z 840.6220). To confirm the identity of the $C_{18:1}$-C_6–NBD–DHS species we performed structural analysis by ion-trap MS/MS. The fragment ions derived from the ion

with m/z 840.6215 could all be ascribed structural features in the intact acylceramide ion (Supplementary Figure S2 at http://www.BiochemJ.org/bj/447/bj4470103add.htm). Fragmentation thus confirms the synthesis of $C_{18:1}$-C_6–NBD–DHS in WT microsomes incubated with the precursor C_6–NBD–DHS.

Next, to confirm that Lro1p is responsible for the synthesis of $C_{18:1}$-C_6–NBD–DHS, we tested whether increasing the expression level of the enzyme would increase the production of the acylceramide species. To this end, we monitored the production of $C_{18:1}$-C_6–NBD–DHS in microsomes from $are1\Delta are2\Delta dga1\Delta.LRO1$ cells overexpressing $LRO1$ when cultured on galactose and in microsomes from low-level-expressing cells cultured on glucose. We observed that the levels of $C_{18:1}$-C_6–NBD–DHS were massively increased when Lro1p was overexpressed (Figures 5E–5G). In comparison, incubating microsomes from cells overexpressing $DGA1$ with C_6–NBD–DHS did not yield significant amounts of $C_{18:1}$-C_6–NBD–DHS (Figure 5H).

PE and PC, which are the preferred substrates of Lro1p, most often carry $C_{18:1}$ at their sn-2 position [27] and, as shown in Figure 5, this species seems to be attached by microsomes in an $LRO1$-dependent manner to C_6–NBD–DHS. Taken together, these data support the idea that Lro1p is directly involved in transferring the fatty acid from PE or PC to ceramides.

Purified Lro1p can acylate NBD–ceramide *in vitro*

Lro1p–GFP was immunopurified from a strain with the chromosomal $LRO1$ tagged with GFP and placed under the $GAL1$ promoter (Figure 4G). Purified Lro1p–GFP was assayed in the presence of NBD–ceramide and PC as shown in Figure 4(F). Calculating how much NBD–ceramide was transformed into acylceramide per microgram of protein during the assay demonstrated that the ceramide acylating activity was more than 800-fold enriched in the purified Lro1p–GFP fraction as compared with the immunodepleted supernatant. This again strongly suggests that the ceramide acylation reaction is carried out by Lro1p.

Ceramide acylation also occurs in living cells

Many processes taking place *in vitro* have no physiological correlate. Our calculations on the basis of concentrations of free ceramides in cells reported in the literature [4,28] and on the specific activity of [³H]palmitate added to microsomes of WT cells in assays of the type shown in Figure 2(A) led us to conclude that we generated approximately 45–125 pmol of [³H]C_{16}-ceramide in microsomes that already contained a minimum of 50–100 pmol of C_{26}-ceramides. This latter estimate is probably too low, since it can be expected that ceramides are more concentrated in ER-derived Lro1p-containing microsomes than in the average cellular membrane. Also, when doing reverse ceramidase assays with WT.YPC1 microsomes as in Figure 2(A), but not adding non-radioactive palmitate into the assays, so that only 14 pmol of [³H]ceramide was generated, we always found that 25–30 % of ceramides appeared as acylceramides (results not shown). This convinced us that the acylation of [³H]C_{16}-ceramide by microsomes observed *in vitro* (Figure 1A) occurred at ceramide concentrations that were not significantly above the physiological range of ceramides in living cells. To gain further insight and to see also whether C_{26}-ceramides were acylated, we labelled yeast cells with [¹⁴C]serine, allowing us to follow sphingolipid metabolism *in vivo*. WT cells fed with [¹⁴C]serine produced a substantial amount of a non-polar lipid migrating to the position
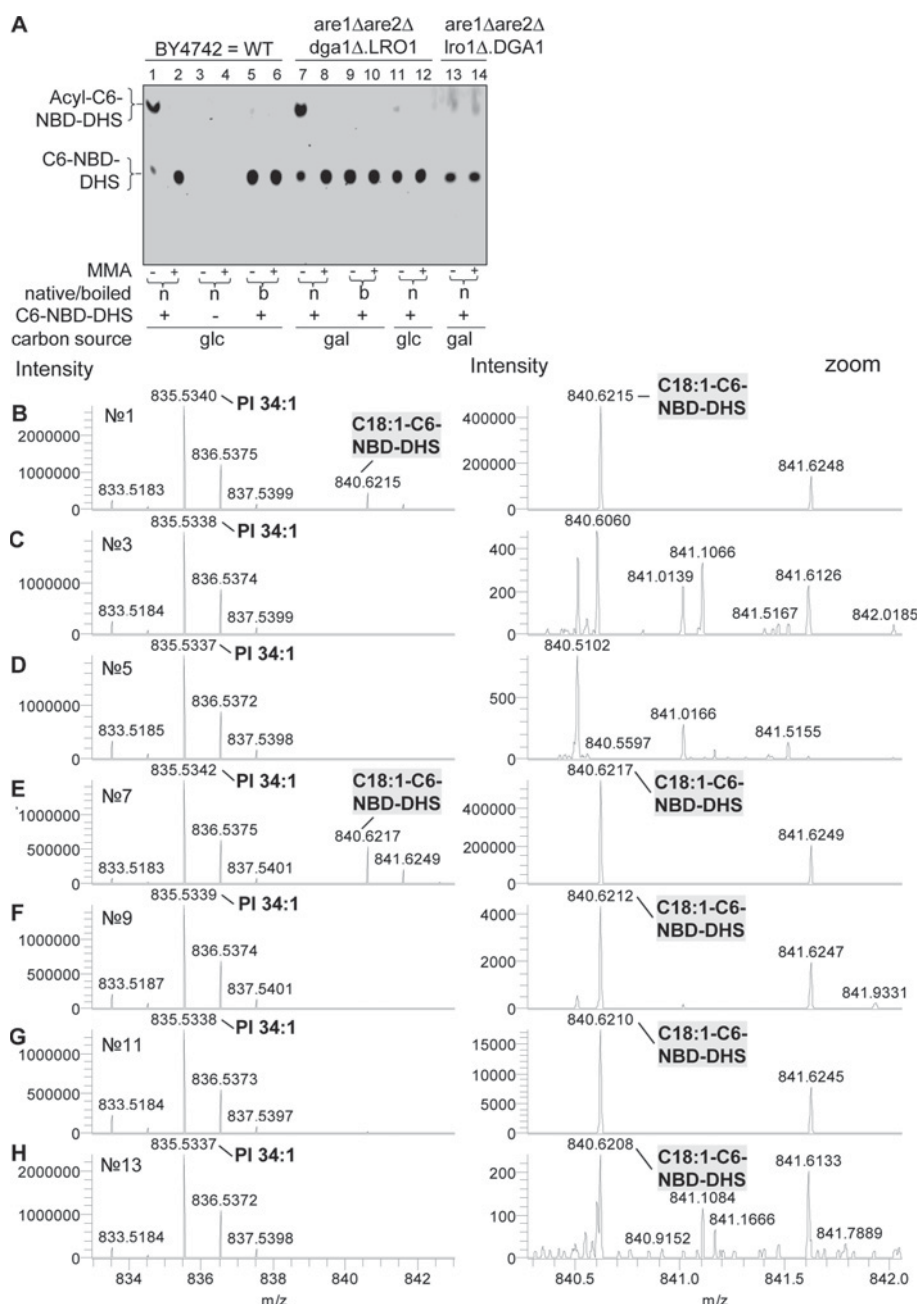
Figure 5   Profiling of $C_{18:1}$-C_6–NBD–DHS by MS

(**A**) The indicated strains were grown on glucose or galactose overnight. Native or boiled microsomes from WT and mutants were incubated for 2 h at 30°C in the presence or absence of C_6–NBD–DHS. Each reaction was split into two equal parts of 20 μl, each containing 25 μg of protein. Lipids extracted from one half were split and treated with methanol or MMA for alkaline hydrolysis and analysed by TLC. b, boiled; gal, galactose; glc, glucose; n, native. (**B**–**H**) Lipid extracts of the second half of the microsomes were subjected to FT-MS analysis as described in the Experimental section. Panels on the right-hand side depict magnified spectra of those on the left-hand side. (**B**–**H**) contain samples corresponding to lanes 1, 3, 5, 7, 9, 11 and 13 of (**A**) as indicated. (**B**) Microsomes from WT cells incubated with C_6–NBD–DHS; (**C**) microsomes from WT cells without the addition of C_6–NBD–DHS; (**D**) microsomes from WT cells, boiled and incubated with C_6–NBD–DHS; (**E**) microsomes from *are1△are2△dga1△.LRO1* cells cultured in YPG incubated with C_6–NBD–DHS; (**F**) microsomes from *are1△are2△dga1△.LRO1* cells cultured in YPG, boiled and incubated with C_6–NBD–DHS; (**G**) microsomes from *are1△are2△dga1△.LRO1* cells cultured in YPD incubated with C_6–NBD–DHS; and (**H**) microsomes from *are1△are2△lro1△.DGA1* cells cultured with YPG incubated with C_6–NBD–DHS.

of acylated ceramide (Figure 6A, asterisk). Mild base treatment led to the disappearance of part of this lipid and the appearance or increases of lipids considered to represent DHS-C_{26} and PHS-C_{26}-OH (Figure 6A, lanes 7 and 8). In many cases hydrolysis of these presumably acylated ceramides was incomplete, in others it was complete and we presently are not quite sure whether this hydrophobic lipid is heterogeneous, containing a mild-base-

sensitive and a mild-base-resistant component, or if it is not efficiently solubilized and therefore not efficiently hydrolysed.

As shown in Figure 6(A), we labelled the living cells with [^{14}C]serine for various periods of time allowing us to observe the kinetics of acylceramide synthesis (Figure 6A). The level of [^{14}C]ceramides reached steady-state levels after 40 min and thereafter slowly declined (Figure 6B). Acylceramides increased
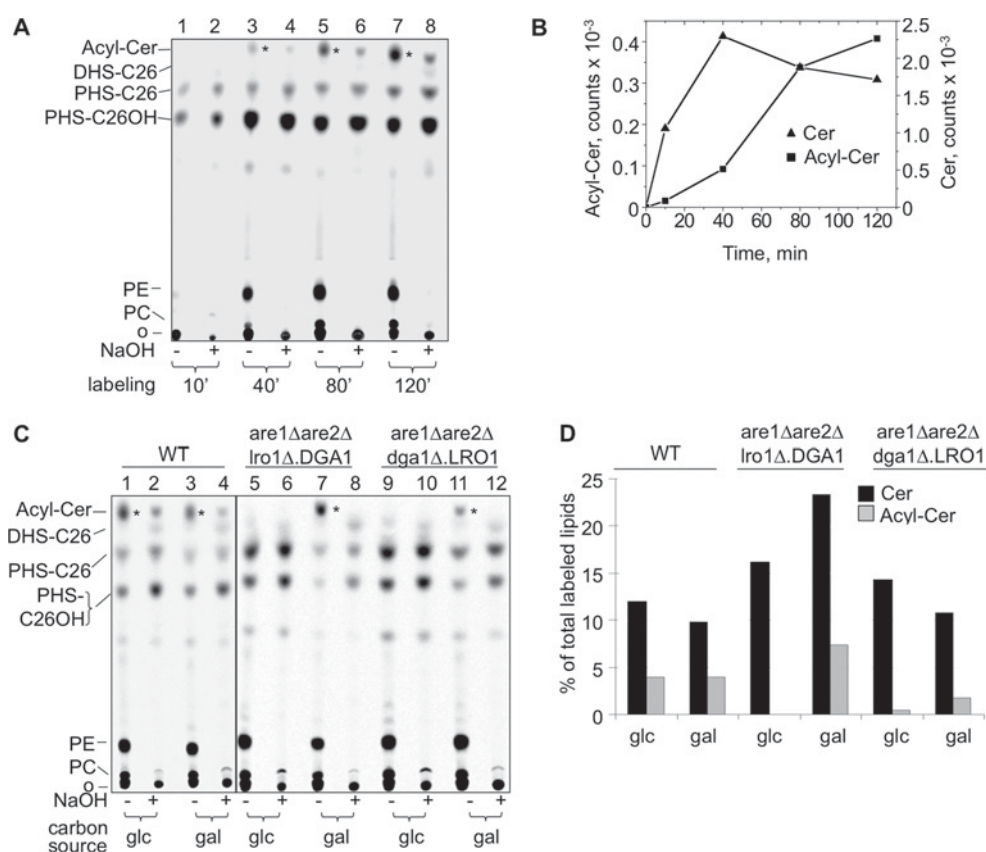
Figure 6 *DGA1* and *LRO1* make acylceramides *in vivo*

(**A**) WT cells were metabolically labelled with [^{14}C]serine for 10–120 min. Lipids were extracted, treated with sodium hydroxide or mock-incubated and separated by TLC; o, origin. The assignment of DHS-C$_{26}$, PHS-C$_{26}$ and PHS-C$_{26}$-OH to distinct bands in this and following Figures is tentative and solely based on the presence or absence of major bands of the same mobility in *sur2Δ*, *scs7Δ* and *sur2Δscs7Δ* cells lacking the LCB-C$_4$-hydroxylase, the fatty acid C$_2$-hydroxylase or both [31] (results not shown). Assignments are supported by previous data on the relative abundance of the various IPC and ceramide species in yeast [18,30,32]. The TLC system used is mainly sensitive to the number of hydroxy groups of ceramides, not fatty acid chain length. (**B**) Amounts of acylceramides and the sum of all ceramides from (**A**) were obtained by radioscanning and plotted as c.p.m. against time of labelling. (**C**) WT, *are1Δare2Δlro1Δ.DGA1* and *are1Δare2Δdga1Δ.LRO1* cells were grown overnight on glucose (glc) or galactose (gal) and then labelled with [^{14}C]serine at 30°C for 160 min on the same carbon source as used in the preculture. Growth on galactose led to a drastic induction of Lro1p and Dga1p (Supplementary Figure S3 at http://www.BiochemJ.org/bj/447/bj4470103add.htm). Lipids were extracted, desalted and deacylated with sodium hydroxide or were mock-incubated. They then were resolved by TLC. (**D**) Ceramides and acylceramides in (**C**) were quantified by radioscanning. Cer, ceramide; gal, galactose; glc, glucose; o, origin.

at maximal speed only after 40 min, when ceramides had reached their maximal level but did not reach a plateau during the 2 h of labelling (Figure 6B). These data are compatible with a continuous acylation of part of the newly made ceramides. The same amounts of acylceramide were also observed, when *ypc1Δydc1Δ* cells were metabolically labelled, suggesting that the bulk of ceramides that are acylated *in vivo* are made by Lag1p and Lac1p (results not shown).

To see whether Lro1p, and possibly Dga1p or Are2p, were involved in making the very hydrophobic lipid species observed in Figure 6(A), we again used the *are1Δare2Δlro1Δ.DGA1*, *are1Δare2Δdga1Δ.LRO1* and *are1Δlro1Δdga1Δ.ARE2* strains, in which the endogenous *DGA1*, *LRO1* or *ARE2* genes have been placed behind the *GAL1*-promoter respectively. These mutants have no LDs when grown on glucose, but induce LDs on galactose, indicating that expression of one of the three genes *LRO1*, *DGA1* or *ARE2* is sufficient to cause the appearance of LDs [12,16]. Accordingly, these strains were grown on glucose or on galactose and then labelled with [^{14}C]serine along with WT cells (Figure 6C). *LRO1* and *DGA1* overexpression was verified by Western blotting (Supplementary Figure S3 at http://www.BiochemJ.org/bj/447/bj4470103add.htm) and allowed the cells to produce significant amounts of acylceramides

(Figure 6C, lanes 5–12, and Figure 6D). *ARE2* overexpression did not have any effect on the conversion of ceramide into its esters (results not shown), although its overexpression leads to a rapid appearance of LDs in an *are1Δare2Δlro1Δdga1Δ* background [29].

Significantly, all of these cell lines growing on glucose made very few acylceramides. This indicates that Lro1p and Dga1p are the only enzymes that can acylate the ceramide in living cells.

The quantification of the presumed acylceramides was always done by subtracting mild-base-resistant counts from total counts in the acylceramide band of the untreated sample (see the Experimental section). Calculated in this way, 7% of the total incorporated radioactivity was present as acylceramide when *DGA1* was overexpressed, and only 2% when *LRO1* was overexpressed (Figure 6D). In WT cells acylceramides amounted usually to approximately 2–5% of total labelled lipids. In other words, a quarter of the total ceramides was usually esterified in WT cells (Figure 6D).

Characterization of [^{14}C]serine-labelled acylceramides

To obtain additional evidence for the biosynthesis of acylceramide *in vivo* we also labelled cells with [^{14}C]serine in the presence of
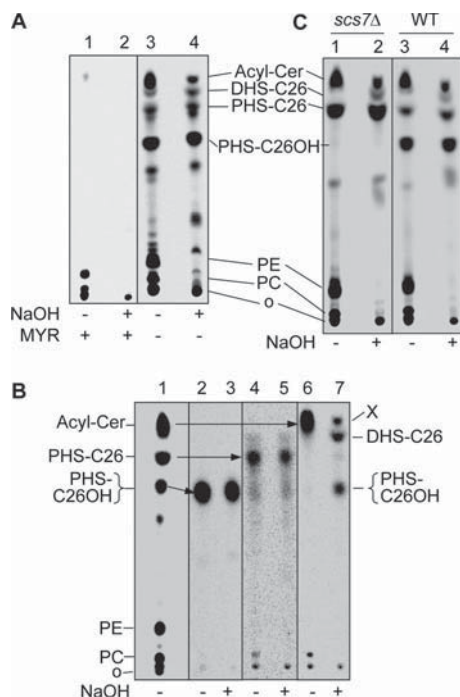
Figure 7   The main ceramides acylated in WT cells *in vivo* co-migrate with DHS-C$_{26}$ and PHS-C$_{26}$-OH

(**A**) WT cells were cultured with or without 40 μg/ml myriocin (MYR) for 2 h. Then the cells were labelled with [^{14}C]serine for 160 min at 30 °C in the same medium as used for pre-incubation. The extracted lipids were treated and resolved by TLC. (**B**) WT cells were grown at 30 °C and labelled with [^{14}C]serine for 2 h at the same temperature. The labelled lipids were resolved by TLC resulting in the profile in lane 1. The main ceramides and acylceramides were scraped off of the TLC plate, extracted from the silica and subjected to mild base hydrolysis with sodium hydroxide or mock-treated. Horizontal arrows connect identical lipids before and after scraping. The sodium-hydroxide-resistant lipid co-migrating with acylceramide is marked as 'X'. The Figure shows one of three experiments which gave similar results. The quantification of all three experiments is given in Table 3. (**C**) *scs7Δ* and WT cells were labelled with [^{14}C]serine and lipid extracts were analysed by TLC. Cer, ceramide; o, origin.

Table 3   Quantification of deacylation products of [^{14}C]serine-labelled acylceramides

The abundance of different lipids in lane 7 of Figure 7(B) and in two similar experiments is expressed as a percentage of total lipids after mild base treatment.

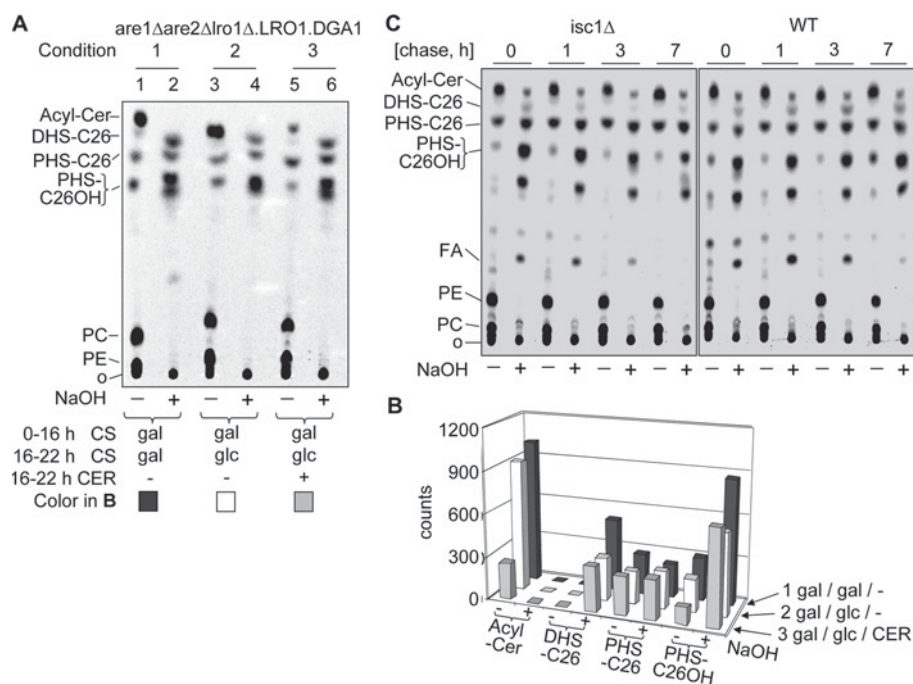|	Experiment number			
Deacetylation product	1	2	3	1–3 mean (S.D.)
X (sodium-hydroxide-resistant)	23.1%	38.9%	27.7%	29.9 % (8.1)
DHS-C$_{26}$	37.9%	37.9%	15.4%	30.4 % (13.0)
PHS-C$_{26}$-OH	38.9%	23.3%	56.9%	39.7 % (16.8)

a large fraction of which was hydrolysed to lipids running with DHS-C$_{26}$ and PHS-C$_{26}$, the latter being the main ceramide of this strain [31]. Thus the deletion of *SCS7* does not prevent the biosynthesis of acylceramides and suggests that, although PHS-C$_{26}$ is not efficiently acylated in WT cells, the acyltransferases can utilize it in a *scs7Δ* background and that the resulting acylated PHS-C$_{26}$ is stable.

In view of the variability of the relative amounts of acylceramides observed in our experiments and in view of the known ability of yeast cells to secrete apolar lipids, such as acetylated cholesterol [33], we tested whether yeast cells would secrete any acylceramide. However, when analysing the growth medium we could not detect any secreted acylceramides (Supplementary Figure S4A at http://www.BiochemJ.org/bj/447/bj4470103add.htm).

Acylceramides can be mobilized when fatty acid synthesis is compromised

We also wondered whether an increased demand for fatty acids inducing the disappearance of LDs could lead to the hydrolysis of acylceramides. To test for this we utilized conditions in which high amounts of acylceramide were available for degradation. *DGA1* and *LRO1* were simultaneously induced in *are1Δare2Δlro1Δ.DGA1.LRO1* cells in galactose-containing medium and cells were at the same time labelled with [^{14}C]serine for 16 h (Figure 8A). Chasing in fresh glucose medium, allowing for repression of *DGA1* and *LRO1*, did not result in mobilization of acylceramides as compared with the simple continuation of the culture in the labelling medium (Figure 8A, condition 2 compared with condition 1, and Figure 8B). On the other hand, chasing with glucose medium supplemented with the fatty acid synthase inhibitor cerulenin led to the almost complete disappearance of acylceramides within 6 h (Figure 8A, condition 3, and Figure 8B). It is worth noting that, in Figure 8, acylceramides were labelled in the absence of cycloheximide. Indeed, this drug enhances incorporation of [^{14}C]serine into lipids, but acylceramides are also made in its absence (Supplementary Figure S4B).

We also tried to force the mobilization of acylceramides by inflicting a want of sphingolipids. For this, cells were labelled with [^{14}C]serine for 12 h and chased for the indicated periods with non-radioactive serine in the presence of myriocin (Figure 8C). WT cells were used in this experiment as well as a strain deficient in Isc1p, the only enzyme in yeast able to hydrolyse IPCs [34,35]. In *isc1Δ* mutants the pathway generating ceramides via the breakdown of complex sphingolipids is blocked (Figure 1). TLC analysis did not show any significant mobilization of acylceramide during chase in both WT and *isc1Δ* (Figure 8C) cells, and the quantification by Berthold scanning confirmed this impression (Supplementary Figrues S5A and S5B at http://www.BiochemJ.org/bj/447/bj4470103add.htm, red

myriocin, a specific inhibitor of the serine palmitoyl transferase catalysing the first step of sphingolipid biosynthesis. The synthesis of ceramides and acylceramide was strongly repressed by myriocin (Figure 7A). This further confirmed that the very hydrophobic labelled lipids observed after metabolic labelling with [^{14}C]serine are indeed derived from sphingolipids, the biosynthesis of which requires *LCB1*. A similar reduction of ceramide and acylceramide biosynthesis was also observed in the thermosensitive lcb1-100 cells, labelled at 37 °C (results not shown).

Yeast ceramides either contain DHS or PHS, and their fatty acid can be either non-, mono- or bis-hydroxylated [30,31]. The most abundant ceramide of yeast is phytoceramide containing an α-hydroxylated C$_{26:0}$ fatty acid (PHS-C$_{26}$-OH) [18,32]. To find out what ceramides became acylated, and to corroborate the idea that the most hydrophobic [^{14}C]serine-labelled lipids in Figure 6 are acylceramides, such lipids were scraped off of the TLC plate and treated with mild base. Acylceramide from WT cells contained on average 40 % of a lipid running with PHS-C$_{26}$-OH and 30 % of a lipid running with DHS-C$_{26}$, the remainder being mild base resistant for unknown reasons (see above) (Figure 7B and Table 3). To see whether PHS-C$_{26}$-type ceramides can also be acylated, we also labelled the *scs7Δ* strain, lacking the fatty acid hydroxylase [31] (Figure 7C). Metabolic labelling of *scs7Δ* with [^{14}C]serine yielded a major band in the region of acylceramides,

Figure 8   Disappearance of acylceramides under drug-induced lipid deprivation

(**A**) *are1Δare2Δdga1Δ.DGA1* cells bearing *DGA1* behind the *GAL1* promoter were grown overnight to a D_{600} of 3.0 in galactose medium while being labelled with [¹⁴C]serine at 30°C (16 h labelling). On the next day, the cell culture was split into three equal parts and treated as follows. Condition 1, culture of cells in the presence of [¹⁴C]serine was simply continued for a further 6 h without changing the medium; condition 2, cells were collected, washed and further incubated in an equal volume of fresh medium without [¹⁴C]serine containing glucose as a carbon source (CS) for 6 h; condition 3, as for condition 2, but the medium was supplemented with 10 μg/ml cerulenin (CER). Extracted lipids were resolved by TLC. (**B**) Quantification of data in (**A**) was expressed as absolute counts present in the various lipid species on the Berthold scan. (**C**) WT and *isc1Δ* cells were labelled with [¹⁴C]serine for 12 h at 30°C to a D_{600} of 8.0. After labelling, cells were collected, washed and resuspended at a D_{600} of 1.0 in YPD medium supplemented with 7 mg/ml serine and 40 μg/ml myriocin. Cells were chased at 30°C and a fixed volume of culture was removed for lipid analysis after 0, 1, 3 or 7 h. Lipids were extracted and treated as described above. Quantification of some lipid species during chase are shown in Supplementary Figure S5 (at http://www.BiochemJ.org/bj/447/bj4470103add.htm). Cer, ceramide; gal, galactose; glc, glucose; o, origin.

columns). Indeed, the lipid profiles in WT and *isc1Δ* cells were very similar. Thus it appears that an acute lack of sphingolipids does not induce a mobilization of the ceramide moiety of acylceramides.

DISCUSSION

This present paper discusses the existence of a metabolic pathway in yeast, which previously had been characterized in mammalian cells. It allows ceramides to not only be processed for IPC biosynthesis or degradation via the ceramidases, but also to be transformed into acylceramides as shown in Figure 1. Acylceramides, as judged by their physicochemical properties, could well be deposited in LDs, although this will have to be tested experimentally. At the moment it is not clear whether this pathway represents a mere detoxification mechanism to protect cells from toxic amounts of ceramides or fatty acids [36], or whether it also serves a storage purpose, similar to TAGs, which are mobilized to allow for rapid membrane biosynthesis when stationary phase cells are diluted into nutrient-rich media and resume growth [37].

The acylceramides seem to be made by Lro1p and Dga1p, the former being the only enzyme showing activity in our microsomal *in vitro* system. In intact cells Dga1p also seems to be able to acylate ceramides, according to metabolic labelling experiments. It thus appears that Dga1p and Lro1p can utilize ceramide instead of DAG, but it is difficult to estimate the relative contributions of these enzymes from our experiments, because

the demonstration that either enzyme can acylate ceramides in living cells was performed in cells overexpressing either Lro1p or Dga1p (Figure 6C).

Dga1p and Lro1p were previously shown to have relaxed substrate specificity in that they can also use monoacylglycerol and long-chain alcohols as an acceptor substrate [38,39], although they cannot use ergosterol [16,40]. It has been reported that in microsomal assays Lro1p uses PE and PC as donor substrates, transfers saturated, unsaturated and even the non-natural polyunsaturated fatty acids groups and may transfer fatty acids also from the *sn*-1 position, albeit at an approximately 10-fold lower rate than from the *sn*-2 position; it can even use DAG as a donor substrate [15,38].

We were able to show that microsomes attach O-acyls to the position 1 of sphingosine-containing ceramides using DDQ oxidation (Figure 4). We tried to confirm this result by testing whether ceramides lacking the hydroxy group on C1 or lacking the hydroxylated C1 atom altogether could still be acylated. For this we added to microsomes [³H]C₁₆:₀ plus 1-deoxysphinganine or 1-deoxymethylsphingosine, lacking the terminal OH- or OH-CH₂- group of classical LCBs, but these LCB analogues were not recognized as substrates by Ypc1p, and no corresponding 1-deoxy- or 1-deoxymethyl-[³H]ceramides were generated in our microsomal *in vitro* system (results not shown). Ypc1p also did not recognize 3-oxo-sphinganine (results not shown). Thus we could not confirm that the O-acylation of DHS- and PHS-containing ceramides occurs on C1 using these approaches.

In our TLC system the deacylation products of the *in-vivo*-generated acylceramides mainly ran at the positions of

DHS-C_{26} and PHS-C_{26}-OH, whereas almost no material co-migrating with PHS-C_{26}-OH appeared upon deacylation of acylceramides of the *scs7Δ* mutant lacking fatty acid hydroxylase (Figure 7C). Although this suggests that Dga1p and Lro1p mainly acylate DHS-C_{26} and PHS-C_{26}-OH, other ceramides may run to the same position on TLC. For instance, the position in this TLC system of the previously described PHS-C_{14}-OH species [32] is unknown. Clearly more studies are required to identify the exact nature of the ceramide species acylated in living cells.

Lro1p is the only yeast homologue of the mammalian LPLA2, the first and only other enzyme reported to acylate ceramides on C1 [23,25,41]. The enzyme is a group XV PLA$_2$, and is a close homologue of the extracellular LCAT of high-density lipoproteins. Although LPLA2 seems to function predominantly as a lysosomal acid phospholipase, it can, as many phospholipases, also act as a transacylase in the presence of suitable acceptor substrates. As (donor) substrates it prefers PE and PC and, contrary to its name, can release fatty acids from both *sn*-2 and *sn*-1 positions [42]. The acceptor specificity of LPLA2 is broad, and many long-chain hydrocarbons possessing a primary alcoholic group can be used as substrates [23]. As far as ceramides are concerned, the best substrate in *in vitro* reactions was *N*-acetyl-sphingosine, and much less activity was found with *N*-octanoyl- or *N*-stearyl-sphingosine as substrates [25]. Whether LPLA2 *in vivo* uses conventional ceramides with fatty acids of 14–24 carbon atoms is not clear.

Lro1p, LCAT and PLAL2 are homologues and, according to the conserved domains database at NCBI (http://www.ncbi.nlm. nih.gov/cdd/), they all have numerous identities with the 377 residues long pfam02450 consensus sequence over its entire length. Lro1p, LCAT and LPLA2 all contain a catalytic serine-histidine-aspartate triad; the first two in addition also contain a serine lipase motif [VxL(I/V)GH**S**xG] [13,43]. In analogy to LCAT and LPLA2, which are active extracellularly and in the lysosome respectively, the catalytic residues of Lro1p also lie outside the cytoplasm, namely in the ER lumen. Indeed, Lro1p consists of a type II integral membrane glycoprotein with a single transmembrane helix [44]. Ceramides and DAGs therefore need to reach the ER lumen in order to get access to its catalytic site.

Metabolic labelling with [^{14}C]serine suggests that some newly made ceramides may be channeled into acylceramide biosynthesis and that they can be remobilized in the presence of cerulenin (Figure 8). It is not clear at present what enzymes hydrolyse the O-acyl group of acylceramides, and also it cannot be decided whether cerulenin mobilizes acylceramides by activating certain hydrolases or whether the mobilization is simply due to the disappearance of LDs [12]. Disappearance of LDs might bring acylceramides into contact with ER-localized hydrolases. Whatever the mechanism, the fact that acylceramides remain stable in the absence of cerulenin but start to disappear in its presence (Figure 8A, lanes 1 and 2 compared with lanes 5 and 6, and Figure 8B) does suggest that acylceramide breakdown may somehow be regulated.

AUTHOR CONTRIBUTION

Natalia Voynova and Christine Vionnet carried out most of the experimental work. Christer Ejsing performed and interpreted the mass spectrometric analysis. Natalia Voynova and Andreas Conzelmann designed the experiments and wrote the paper.

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SUPPLEMENTARY ONLINE DATA
A novel pathway of ceramide metabolism in *Saccharomyces cerevisiae*

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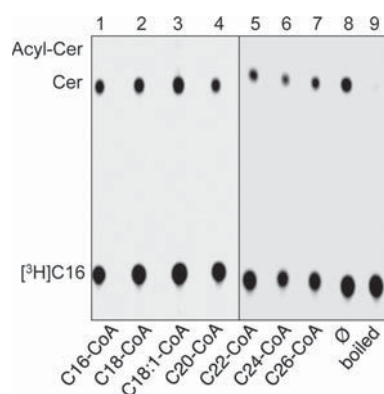
Figure S1 Dga1p does not contribute significantly to microsomal acylceramide synthesis *in vitro*

Microsomes of *Iro1Δ* cells overexpressing *YPC1* (Iro1Δ.YPC1) were incubated with PHS and [³H]palmitate to allow Ypc1p-driven ceramide synthesis to take place as in Figure 2(A), lane 8 of the main text. After a 1 h incubation, the reaction mixture was supplemented with different acyl-CoAs as indicated and samples were incubated for a further 1 h in conditions that are appropriate for Dga1p-mediated TAG synthesis. Lipids were extracted and resolved by TLC. Boiled microsomes were used for the incubation shown in lane 9. Cer, ceramide.
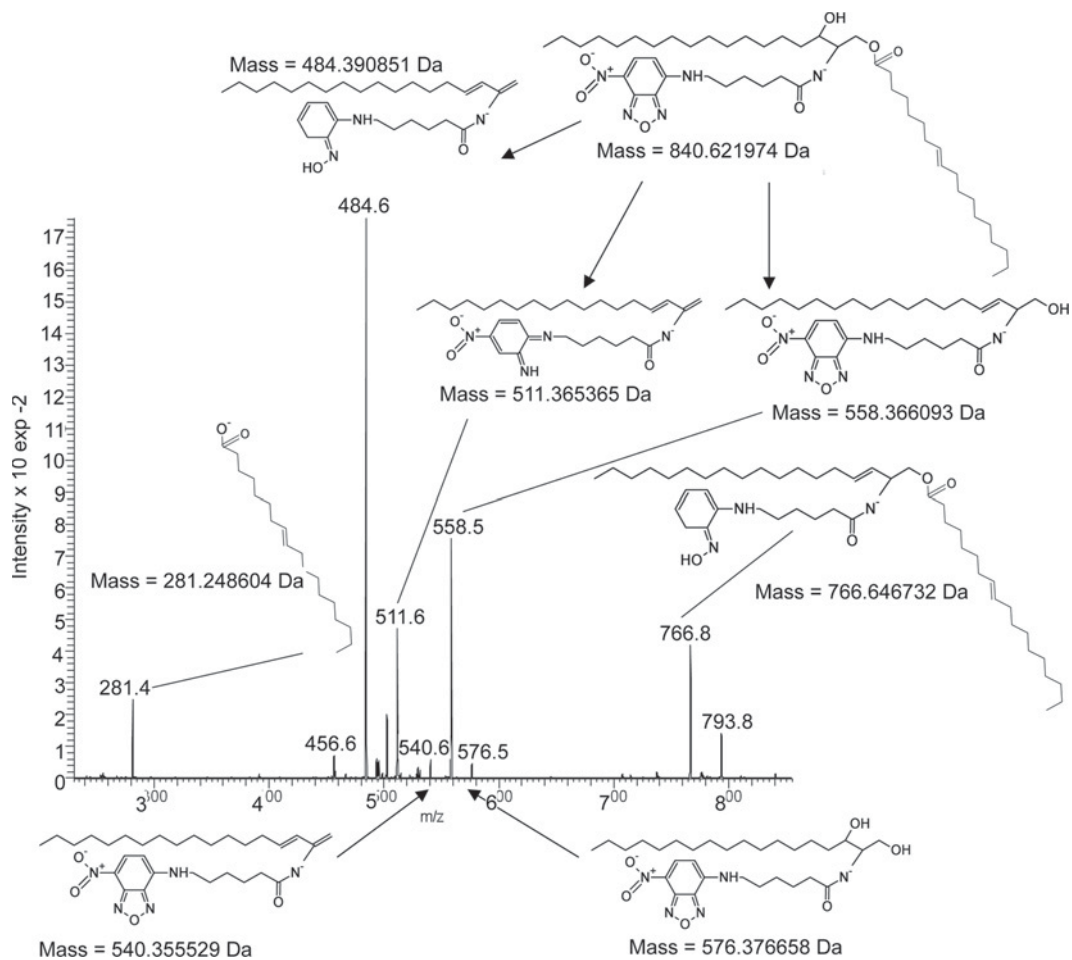
Figure S2   Structural analysis of $C_{18:1}$-C_6–NBD–DHS by ion-trap MS/MS

$C_{18:1}$-C_6–NBD–DHS ion at m/z 840.6215 detected in microsomes from wild-type cells incubated with C_6–NBD–DHS (Figure 5B of the main text) was subjected to collision-induced dissociation. The m/z of fragment ions in the resulting MS/MS spectrum confirm the identity of the $C_{18:1}$-C_6–NBD–DHS molecule as outlined in the fragmentation scheme.
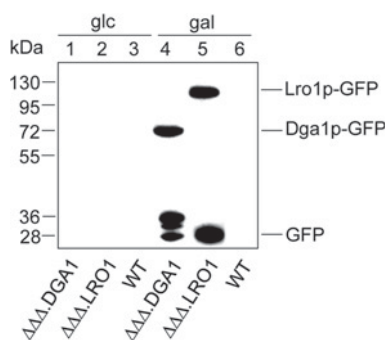
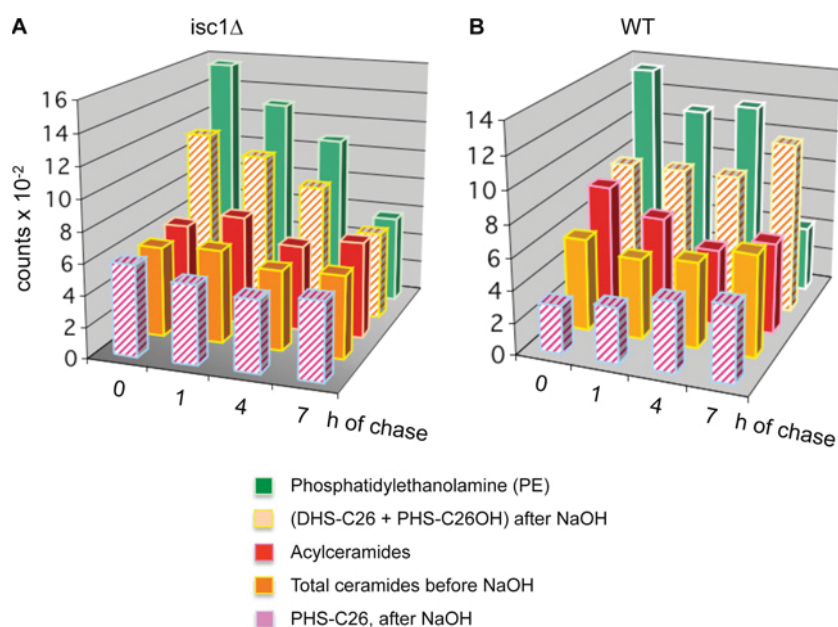**Figure S3   Galactose induces overexpression of *LRO1* or *DGA1***

Total cell lysate was prepared from *are1△are2△lro1△.DGA1* (△△△.DGA1), *are1△are2△dga1△.LRO1* (△△△.LRO1) and WT strains grown overnight in galactose for the experiment shown in Figure 6(C) of the main text. The proteins were resolved by SDS/PAGE and detected by Western blotting using an anti-GFP antibody. The bands at 102 kDa and 74 kDa correspond to Lro1p–GFP and Dga1p–GFP respectively. The molecular mass in kDa is indicated. gal, galactose; glc, glucose.

14
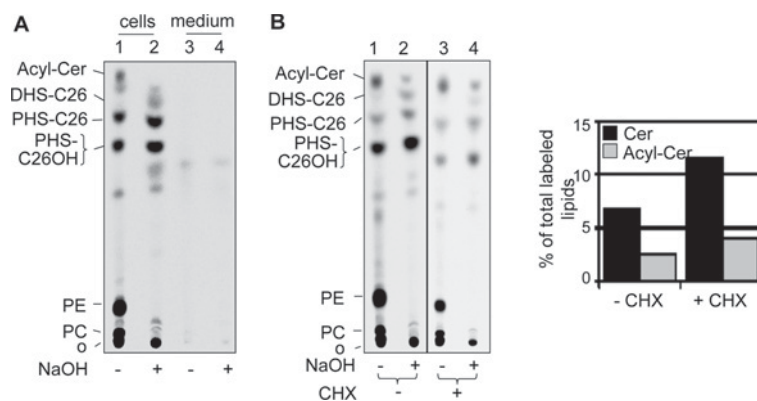
Figure S4   Acylceramides are not secreted and also made in the absence of cycloheximide

(**A**) Cells do not secrete acylceramides. WT cells were labelled with [^{14}C]serine in the presence of 5 mg/ml fatty-acid-free BSA for 160 min. The labelled lipids were extracted from cells and from medium; extracts were desalted, deacylated or not with sodium hydroxide and resolved by TLC. The Figure shows one of two experiments, which gave identical results. (**B**) CHX does not change the lipid profile of cells labelled with [^{14}C]serine. WT cells were labelled with [^{14}C]serine in medium supplemented with or without 10 μg/ml CHX for 160 min at 30°C. The extracted lipids were treated or not with mild base and resolved by TLC. The relative amounts of acylceramides and ceramides are shown on the right-hand side. Cer, ceramide; o, origin.

- ■ Phosphatidylethanolamine (PE)
- ▨ (DHS-C26 + PHS-C26OH) after NaOH
- ■ Acylceramides
- ■ Total ceramides before NaOH
- ▨ PHS-C26, after NaOH

Figure S5   Quantification of TLC shown in Figure 8(C) of the main text

Berthold radioscanning yielded counts for each labelled species after 0, 1, 4 and 7 h of chase in the presence of myriocin. Counts were plotted without any background subtraction except for acylceramides, for which the counts in the mild-base-resistant lanes at the R_f of acylceramides and the R_f of fatty acids were subtracted. (**A**) *isc1*Δ cells. (**B**) WT cells.

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