Lcb4p sphingoid base kinase localizes to the Golgi and late endosomes

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Abstract **Sphingoid long chain base phosphates (LCBPs) regulate cell proliferation, survival and motility in mammals. To learn more about LCBPs in *Saccharomyces cerevisiae*, we determined the cellular location of Lcb4p, the major enzyme catalyzing LCBP synthesis. By indirect immunofluorescence microscopy and subcellular fractionation, Lcb4p localizes to the *trans*-Golgi network and late endosomes and cycles between these compartments. Lcb4p faces the cytosol and is probably bound to membranes by protein–protein interactions. These results indicate that LCBs made in the endoplasmic reticulum must transit to the Golgi to be converted into LCBPs, which must then return to the endoplasmic reticulum to be degraded.**

Key words: Sphingosine kinase; Sphingosine-1-phosphate; Sphingolipid; Phytosphingosine

1. Introduction

Phosphorylated species of sphingoid long chain bases (LCBPs) have been identified in all eukaryotes examined. The major LCBP in mammals is sphingosine-1-phosphate, an important signal for regulating multiple processes including proliferation, survival, motility, cytoskeletal mechanics and differentiation (reviewed in [1,2]). Sphingosine-1-phosphate formation in humans is catalyzed by two related sphingosine kinases, SPHK1 and SPHK2 [3], that are members of a family of enzymes found in all eukaryotes. Conservation of LCBPs and the enzymes that catalyze their formation suggest that LCBPs perform important, perhaps conserved, functions in all organisms. However, studies using *Saccharomyces cerevisiae* have yet to identify specific functions for LCBPs and mutant strains that lack LCBPs display near-normal behavior [4]. To gain insight into the function of LCBPs we sought to determine their site or sites of synthesis by determining the cellular location of Lcb4p, the major long chain base (LCB) kinase in yeast.

S. cerevisiae cells have two kinases, Lcb4p and Lcb5p [4], that phosphorylate dihydrosphingosine (DHS) and phytosphingosine (PHS) to yield DHS-P and PHS-P. Lcb4p accounts for 97% of total cellular LCB kinase activity and Lcb5p accounts for the remainder. Since DHS-P and PHS-P are degraded by two phosphatases, Lcb3p and Ysr3p, and by the Dpl1 lyase, all of which are located in the endoplasmic reticulum (ER) [5,6], it seemed likely that Lcb4p and Lcb5p would also be in the ER. However, we find that most of the Lcb4p is in the Golgi apparatus and in late endosomes.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this study are parental strain RCD224 (*MATa leu2-3, 112 ura3-52 trp1 his4 rme1* [7]) and derivatives of it including RCD220 (*MATa*) and RCD221 (*MATα*) which carry *lcb4-Δ1::KAN lcb5-Δ1::KAN*. *VPS27* was deleted in strain BY4741 (*Sacchoromyces* genome deletion project Stanford University: *MATa his-3Δ1 leu2Δ0 met 15Δ0 ura3Δ0 vps27::KAN*). This strain was crossed to RCD221 to yield RCD388 (*MATa leu2Δ ura3Δ rme1 met15Δ his⁻ lcb4-Δ1::KAN lcb5-Δ1::KAN vps27::KAN*). Yeasts were grown in yeast extract, peptone, dextrose (YPD) [8] or defined medium (SD) containing 0.134% yeast nitrogen base (Difco), 2% glucose and the supplements decribed previously [8]. SSuc is defined medium with 2% sucrose in place of glucose and S-Ura is medium that lacks uracil.

2.2. Plasmids

pLCB4-26 contains 462 bases of the *LCB4* promoter plus the coding region fused in-frame to three hemagglutinin (HA) epitopes, followed by the *CYC1* terminator. The plasmid was constructed by inserting a polymerase chain reaction (PCR) fragment into the *Bam*H1 and *Pst*I sites of pPAD82 (*CEN4 URA3*, [9]). pLCB4-27 has the *LCB4* coding region cloned downstream of the *GAL1* promoter (between the *Xba*I and *Hin*dIII sites) carried in YCplac33GAL (*CEN4 URA3*, *GAL1* promoter cloned into YCplac33, [10]). The 3′ end of the coding region is tagged with codons for three HA epitopes in the same way as in pLCB4-26.

2.3. Immunoblotting and immunofluorescence microscopy

Cell-free yeast extracts [4] were subjected to SDS–PAGE and Western blotting using polyvinylidene fluoride membranes. Rabbit polyclonal antibodies were made against a peptide (AKSKKELKVHFLENKDKNK) representing residues 423–441 of Lcb4p, were affinity-purified using the peptide as a ligand, and used at a 1/1000 dilution for an overnight incubation. Secondary antibody was goat anti-rabbit-IgG-alkaline phosphatase (Gibco-BRL, #13869-011) diluted 1/1000 and incubated 4 h with immunoblots. The phosphatase substrate was ECF (Amersham Pharmacia Biotech #RPN5785) and the fluorescent signal was quantified by using a Molecular Dynamics Storm imaging system. Antibodies against Vph1p, 3-phospho-glycerate kinase, Dpm1p and a mitochondrial porin were from Molecular Probes.

Indirect immunofluorescence staining of formaldehyde-fixed cells [11] used rat anti-HA antibody (3F10, Boehringer), rabbit anti-

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Abbreviations: DHS, dihydrosphingosine; ER, endoplasmic reticulum; LCB, long chain base; LCBP, long chain base phosphate; PHS, phytosphingosine; PM, plasma membrane; PVC, prevacuolar compartment; SD, defined medium; YPD, yeast extract, peptone, dextrose

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Tlg1p (Dr. S. Munro), rabbit anti-Pep12p (Molecular Probes), rabbit anti-Sec7p (Dr. R. Schekman) and rabbit anti-Pma1p (Dr. R. Serrano). Secondary antibodies were FITC-goat anti-rat IgG (Sigma) or Cy3-goat anti-rabbit IgG (Amersham Pharmacia Biotech).

2.4. Subcellular fractionation and enzyme assay

Yeast cells were broken [4] and the lysate was fractionated at 4°C by sequentially centrifuging at 500×*g* for 5 min (P0.5 and S0.5), 13 000×*g* for 10 min (P13 and S13) and 100 000×*g* for 60 min (P100 and S100) [12]. For the experiments presented in Fig. 3, cells grown to an $A_{600\,nm}$ of 0.7 were converted to spheroplasts, gently lysed [12] and fractionated at 4°C by using the same sequential centrifugation protocol as used for the crude subcellular fractionation procedure. The P100 was resuspended in 2 ml of hypo-osmotic buffer (50 mM Tris–HCl, pH 7.5, 200 mM sorbitol, 1 mM EDTA plus protease inhibitors and loaded on top of a 22–60% (w/w) sucrose step gradient. Gradients were centrifuged at 4°C in a Beckman SW40Ti rotor at 170 000×*g* for 18 h and 16 fractions were collected starting at the top.

Membrane association of Lcb4-3HA was measured by using the P100 membrane fraction suspended in buffer (50 mM Tris, pH 7.5, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml each of leupeptin, pepstatin and aprotinin). Samples of the P100 were adjusted to final concentrations of 0.5 M potassium acetate (pH 4.5), 1 M sodium chloride, 0.1 M sodium carbonate (pH 11) and 5 M urea. Samples were incubated on ice for 30 min, vortexed at high speed for six cycles (30 s vortexing and 1 min on ice), centrifuged at 100 000×*g* for 45 min at 4°C and immunoblotted.

For detergent treatment, the P100 fraction was incubated with 0.5% or 1.0% Triton X-100 for 120 min on ice and either sonicated 30 s with a microprobe (Heat System Ultrasonic) or not sonicated followed by centrifugation at 100 000×*g* for 60 min. The supernatant and pellet fractions were immunoblotted.

Lcb4p and Lcb5p kinase activity was measured as described previously [4]. All protein concentrations were quantified by using a Bio-Rad DC kit with bovine serum albumin as a standard.

2.5. Trypsin sensitivity

RCD220 cells, transformed with pLCB4-26 or pWP1055 [13], were grown in SD to an $A_{600\,nm}$ of 0.7. Equal amounts of cells (120 $A_{600\,nm}$ units) were mixed, converted to spheroplasts [12] and centrifuged to yield a P100 fraction which was suspended in buffer (50 mM HEPES, pH 7.5, 5 mM DTT). Ice-cold P100 was treated with trypsin (0.1 mg/ml) in the presence or absence of 0.5% Triton X-100 for up to 30 min. Reactions were stopped by using trypsin inhibitor type I-S and 1 mM PMSF.

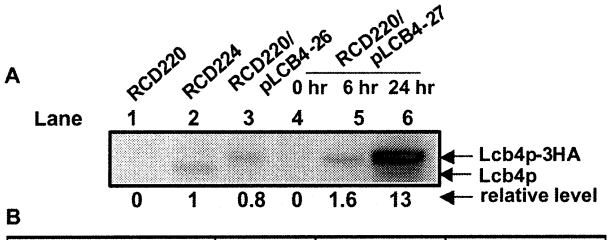
3. Results

3.1. Overproduction of epitope-tagged Lcb4p

About two-thirds of the Lcb4p kinase activity was found to be on membranes [4], an unexpected result, since the protein has no predicted transmembrane domains nor sites for covalent lipid attachment. For localization studies, rabbit polyclonal antibodies were prepared against Lcb4p. Parental RCD224 but not *lcb4Δ lcb5Δ* cells (strain RCD220) showed a band of 75 kDa, the predicted size of Lcb4p, on an immunoblot (Fig. 1A, compare lanes 1 and 2). Thus, the antibody detects Lcb4p; however, specificity is poor and the antibodies react with other proteins (data not shown).

To improve the specificity of detecting Lcb4p, three hemagglutin (HA) epitopes were added to its C-terminus and the tagged gene was expressed on a single-copy vector (pLCB4-26) in *lcb4Δ lcb5Δ* (RCD220) cells under the control of its own promoter. Lcb4p-3HA was detected with anti-Lcb4 antibody as a slightly larger protein of about the same concentration as untagged Lcb4p (Fig. 1, compare lanes 2 and 3). Anti-HA antibody was used in most experiments because detection of Lcb4p-3HA was more specific and sensitive (data not shown).

For some experiments Lcb4p had to be overproduced about



B

Strain/plasmid	Location	Distribution of enzyme units (%)	Specific activity (pmol/mg/min)
RCD224 (WT)	P13 P100 S100	23 45 32	2810 ± 430 2770 ± 350 1550 ± 310
RCD220 (*lcb4Δ lcb5Δ*) (soluble and pellets)		0	0
RCD220/pLCB4-27, 0 hr galactose induction (soluble and pellets)		0	0
RCD220/pLCB4-27, Lcb4p-3HA, 6 hr galactose induction	P13 P100 S100	28 37 35	2100 ± 100 2330 ± 130 1530 ± 340
RCD220/pLCB4-27, Lcb4p-3HA, 24 hr galactose induction	P13 P100 S100	62 30 8	112250 ± 1660 40590 ± 420 7470 ± 250

Fig. 1. Production of HA-tagged Lcb4p in yeast cells. A: Lcb4p was quantified by immunoblotting with Lcb4p antibodies. Extracts analyzed in lanes 1–3 were prepared from cells grown to an $A_{600\,nm}$ of 0.6–0.7 in YPD medium (lanes 1 and 2) or SD-Ura (lane 3). For the samples shown in lanes 4–6 the cells were grown overnight in SD-Ura to an $A_{600\,nm}$ of 0.4, resuspended in S-Ura containing 2% galactose and extracts were prepared after 0, 6 or 24 h incubation. B: LCB kinase specific activity values represent the average of triplicate assays ± S.D. Untransformed RCD224 and RCD220 cells were grown in YPD to an $A_{600\,nm}$ of 1. RCD220/pLCB4-27 cells were grown in SD-Ura medium to an $A_{600\,nm}$ of 1, washed three times with water, resuspended in S-Ura with 2% galactose as the carbon source and grown for 0, 6 or 24 h before cell fractionation and enzyme assay.

two-fold to be detected. Overproduction was achieved by driving transcription of *LCB4-3HA* with the galactose-inducible *GAL1* promoter (pLCB4-27). Cells carrying pLCB4-27 had no detectable Lcb4p-3HA protein when grown in medium containing glucose (Fig. 1, lane 4). Changing the carbon source from glucose to galactose and incubating for 6 or 24 h increased the level of Lcb4p to two- and 13-fold, respectively, above the wild-type level (Fig. 1A, compare lanes 5 and 6 to lane 2). Alternatively, raffinose was used as the carbon source and a 2 h incubation with galactose induced Lcb4p-3HA to about the wild-type level (Fig. 2C).

Neither the presence of the epitope tag nor two-fold overproduction changed the subcellular distribution of Lcb4p-3HA compared to wild-type Lcb4p as measured by kinase activity in subcellular fractions (Fig. 1B, compare values for wild-type RCD224 with *lcb4Δ lcb5Δ* RCD220 cells carrying pLCB4-27, 6 h induction). After 24 h of galactose induction the distribution of Lcb4p-3HA activity changed so that less activity was in the S100 soluble fraction and more was in the P13, plasma membrane (PM) fraction (Fig. 1B).

3.2. Lcb4p localizes to Golgi and endosomes

By indirect immunofluorescence microscopy Lcb4p-3HA was observed both as punctate spots and as diffuse patches in the interior of cells (Fig. 2A), suggesting localization to Golgi or endosomes. To determine which compartment contained Lcb4p-3HA, cells were stained for Sec7p, a late Golgi marker (*trans*-Golgi network [14]), for Tlg1p, found in both

Golgi and early endosomes [12], and for Pep12, found in late endosomes [15,16]. Lcb4p-3HA primarily co-localized with Sec7p (Fig. 2A), indicating localization in the Golgi and to a lesser extent with Tlg1p, indicating co-localization in the Golgi and possibly also in early endosomes. Some co-localization with Pep12p in late endosomes was also seen.

As indicated above, overproduced Lcb4p-3HA tended to localize in the P13 fraction (Fig. 1B, 24 h galactose induction), suggesting binding to the PM. This finding was verified by indirect immunofluorescence microscopy where a fraction of the protein localized to the outer edge of cells where Pma1p, the PM H^+-ATPase, localized (Fig. 2B).

The subcellular location of Lcb4p-3HA was also analyzed by equilibrium centrifugation in a sucrose density gradient. The highest level of endogenous Lcb4p in wild-type cells (RCD224) appeared in fractions that contained the late Golgi marker Sec7p and the late endosomal marker Pep12p (fractions 7 and 8, Fig. 3A). There was less Lcb4p in the early endosomal fractions containing Tlg1p (fractions 11–14, Fig. 3A). In cells producing Lcb4p-3HA there was, in three separate experiments, a consistent separation of fractions containing Sec7p and Pep12p (Fig. 3B), but Lcb4p-3HA was always more abundant in the fraction containing Sec7p (fraction 5, Fig. 3B). Again there was much less Lcb4p-3HA in the early endosomal fractions containing Tlg1p (fractions 12–15, Fig. 3B). These data confirm the indirect immunofluorescence microscopy data (Fig. 2A) and demonstrate that the majority of Lcb4p-3HA is in the Golgi and endosomal compartments.

Many late Golgi proteins in yeast cycle between the late Golgi and late endosomes or the prevacuolar compartment (PVC) [17,18]. Cycling of Lcb4p between these two compartments was examined by using *vps27Δ* cells, which are blocked in transit of proteins from the PVC to the vacuole and the Golgi. *vps27Δ* cells have an enlarged PVC usually located near

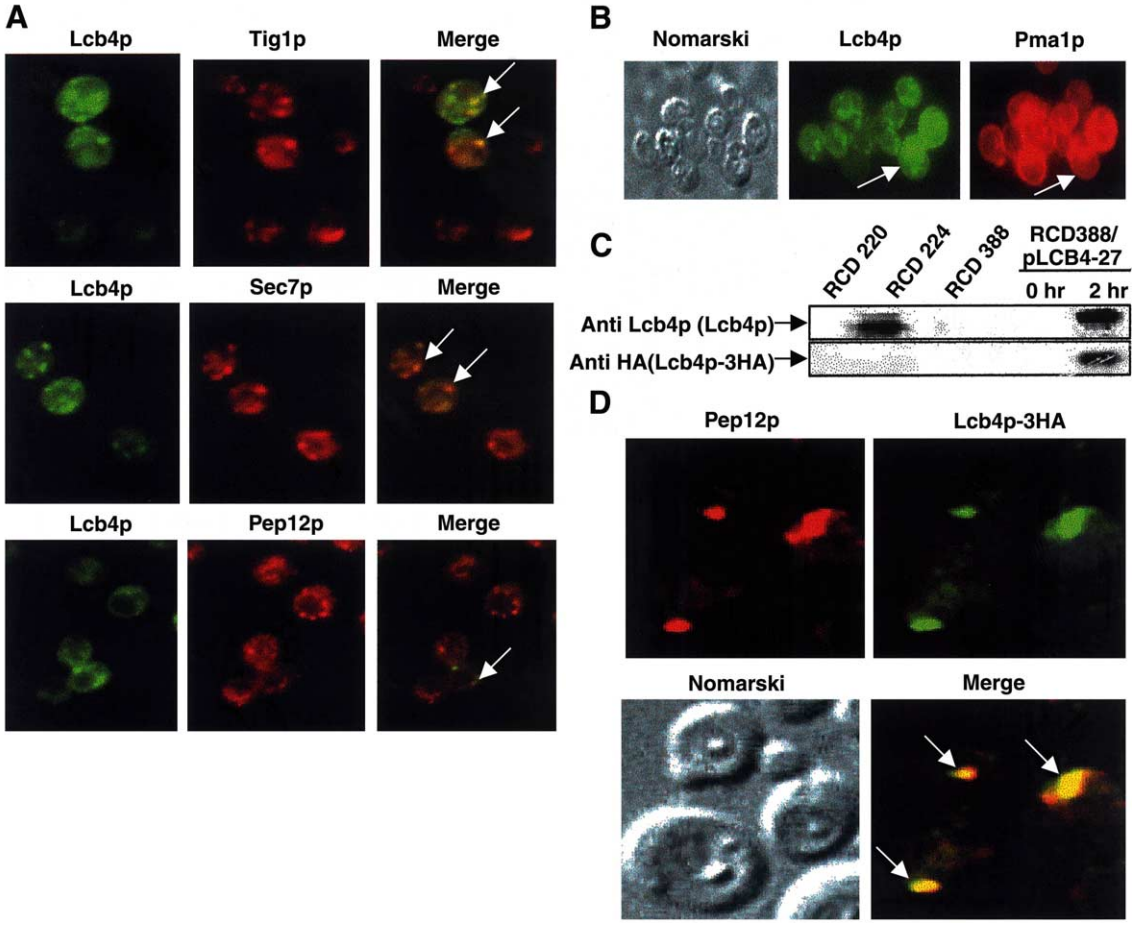


Fig. 2. Lcb4p-3HA localizes to the Golgi apparatus and to early and late endosomes. A: RCD220 (*lcb4Δ lcb5Δ*) cells transformed with pLCB4-27 were grown as described in the legend to Fig. 1 and treated with galactose for 6 h. Samples were processed for immunofluorescent microscopy by treating first with anti-HA antibody (Lcb4p) or with antobodies specific for Sec7p, a Golgi marker, Tlg1p, an early endosomal marker, or Pep12p, a late endosomal marker. Secondary antibodies were coupled to FITC, left panel, or to Cy3, center panels, and the fluorescent signals were merged to identify sites in which Lcb4p co-localized with the marker protein (yellow signals indicated by an arrow, right panels). B: RCD220 (*lcb4Δ lcb5Δ*) cells transormed with pLCB4-27 were grown as described in the legend to Fig. 1 and treated with galactose for 24 h before examination by indirect immunofluorescence microscopy as in panel A. Arrows indicate Lcb4p localization on the PM. A Normarski image is shown at the left. C: The expression level of Lcb4p-3HA in RCD388/pLCB4-27 cells was compared to the level of endogenous Lcb4p in wild-type RCD224 cells and in RCD220 (*lcb4Δ lcb5Δ*) and RCD388 control cells, which both lack Lcb4p. Untransformed cells were grown in S medium containing 2% raffinose and transformed cells were grown in the same medium lacking uracil. Samples immunoblotted with anti-Lcb4p show that the level of Lcb4p-3HA in RCD388/pLCB4-27 cells was induced about two-fold above the wild-type level after 2 h of galactose induction. D: Indirect immunofluorescence microscopy of RCD388/pLCB4-27 cells grown as in panel C and stained first with anti-HA antibody (Lcb4p-3HA) and with anti-Pep12p antibody. Secondary antibodies are the same as in panel A. Arrows indicate overlap of the fluorescent signals and represent the enlarged PVC found in *vps27Δ* cells.

the vacuole [19,20]. In *vps27Δ* cells Lcb4p-3HA was observed in PVC-like structures and co-localized with Pep12p, a marker for late endosomes/PVC [20] (Fig. 2D). These data support the idea that Lcb4p cycles between the late Golgi and late endosomes/PVC in a manner similar to other late Golgi proteins (reviewed in [21]).

3.3. Overproduced Lcb4p accumulates on the PM

The data shown in Figs. 1B and 2B suggest that a portion of overproduced Lcb4p-3HA binds to the PM. To verify this finding and to determine if the protein is enzymatically active, Lcb4p-3HA overproduced in wild-type RCD224 cells was separated into a highly purified PM fraction, a fraction containing other membranes (P150) and a soluble fraction (S150). The purity of fractions was assessed by immunoblotting for marker proteins (Fig. 4, legend). Pma1p was highly enriched in the PM fraction which contained none or very low levels of the other proteins, indicating that the PMs were very pure (Fig. 4A). At time 0 about 60% of endogenous Lcb4p was in the P150 fraction with the rest in the S150 fraction and none in the PM fraction. After 24 h of galactose induction Lcb4p-3HA was overproduced 15-fold and 48% was in the PM fraction, 29% was in the P150 fraction and the rest was in the S150 fraction. These data support the indirect immunofluorescence data (Fig. 2B) and demonstrate that about half of Lcb4p-3HA binds to the PM when the protein is overproduced 15-fold.

Fractions were also assayed for LCB kinase activity (Fig. 4B). At time zero 81% of the enzyme activity was in the P150 fraction of RCD224/pLCB4-27 cells and about 2% was in the PM fraction. After 24 h of galactose induction two-thirds of the activity was in the S150 fraction and only about 1% was in the PM fraction. Because 48% of overproduced Lcb4p-3HA protein (24 h sample) binds to the PM but only has a specific activity three-fold above that in wild-type RCD224 cells of RCD224/pLCB4-27 cells at the 0 h time point, we conclude that Lcb4p bound to the PM is much less enzymatically active than is the enzyme in the P150 and S150 fractions.

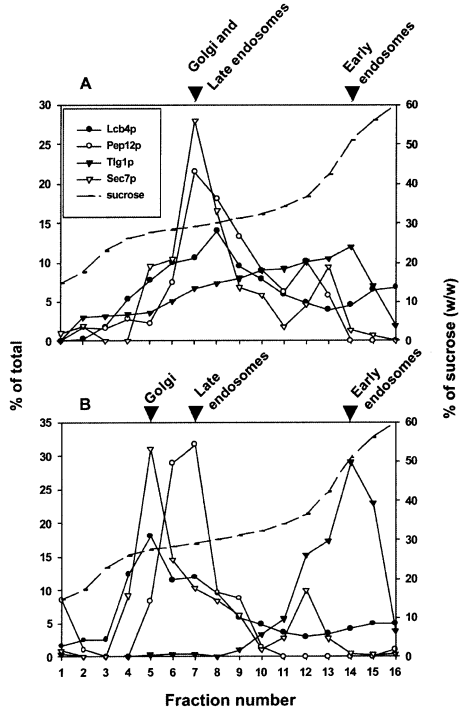
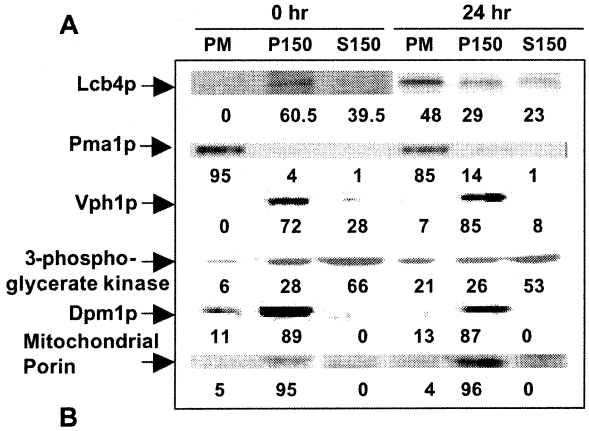


Fig. 3. Localization of Lcb4p by density gradient centrifugation. A: Density gradient profile of the P100 fraction of wild-type RCD224 cells. B: RCD220 cells transformed with pLCB4-27 and induced for 6 h by growth in the presence of galactose. Gradient fractions were analyzed for marker proteins by Western blotting using anti-Sec7p (Golgi), anti-Pep12p (late endosomes) and anti-Tlg1p (early endosomes) antibodies. Proteins in each fraction are expressed as percentages of the total fluorescent signal obtained by immunoblotting. Lcb4p (panel A) and Lcb4p-3HA (panel B) enzyme activity is expressed as a percentage of the total activity on the gradient.



B

Strain	Fraction	Specific activity (pmol/mg/min)	% distribution
RCD224/ pLCB4-27, 0 hr	Lysate	1828 ± 286	100
	PM	3063 ± 158	2
	P!50	3154 ± 274	81
	S150	356 ± 99	17
RCD224/ pLCB4-27, 24 hr	Lysate	19840 ± 159	100
	PM	9829 ± 79	1
	P!50	16777 ± 396	33
	S150	14994 ± 419	66
RCD224 (Wild type)	Lysate	2003 ± 614	100
	PM	2956 ± 70	5 ± 3
	P!50	3420 ± 540	73 ± 12
	S150	537 ± 154	22 ± 9

Fig. 4. Overproduced Lcb4p localizes to the PM and has reduced enzyme activity. A: Untransformed RCD224 cells were grown in YPD to an $A_{600\,nm}$ of 1 and fractionated. Cells transformed with pLCB4-27 were grown in SD-Ura medium to an $A_{600\,nm}$ of 1, washed three times with water, resuspended in S-Ura medium with 2% galactose as the carbon source and fractionated after 0 and 24 h of growth at 30°C. The PM, P150 and S150 fractions (25 μg) were immunoblotted for Lcb4p-3HA, Pma1p (plasma membrane), Vph1p (vacuole), 3-phospho-glycerate kinase (cytosol), Dpm1p (ER) and a mitochondrial porin. The percent distribution indicated below each lane represents the pixels in each band divided by the total pixels in the PM, P150 and S150 bands for each time point. B: Specific activity values for untransformed wild-type RCD224 are the average of three experiments. Values for RCD224/pLCB4-27 cells are for one experiment assayed in triplicate. The percent distribution represents the total Lcb4p kinase activity in each fraction.

3.4. Lcb4p faces the cytoplasm and is tightly bound to membranes

Sensitivity to protease digestion was used to determine if

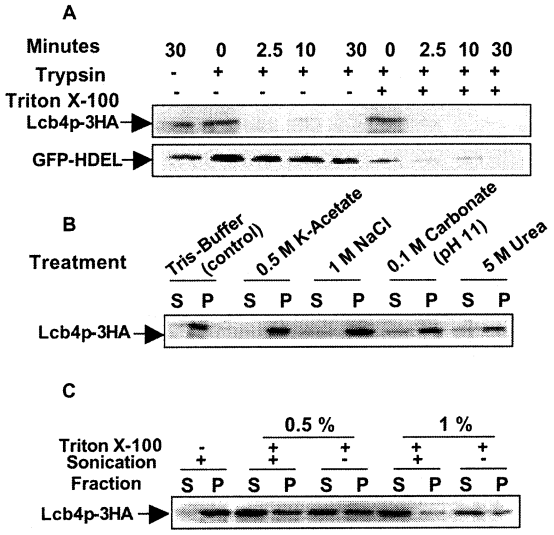


Fig. 5. Lcb4p faces the cytoplasm and binds tightly to membranes. A: Samples were treated as indicated and immunoblotted with anti-HA antibody. B: Following treatment with the indicated agents, samples were centrifuged at 100 000×*g* for 45 min to yield the supernatant fluid (S) and pellet (P), which were immunoblotted. C: Samples treated with Triton and either sonicated or not sonicated were separated into the S and P fractions and immunoblotted.

Lcb3p-3HA is in the lumen or on the cytoplasmic face of Golgi and endosomes. Membranes were treated or not treated with trypsin in the presence or absence of Triton X-100 and immunoblotted. Lcb4p-3HA was sensitive to trypsin digestion even in the absence of Triton (Fig. 5A), in contrast to the lumenal marker protein GFP-HDEL (green fluorescence protein with an HDEL peptide sequence attached to its C-terminus), which was only sensitive to protease digestion in the presence of Triton. Sensitivity to protease digestion in the absence of detergent indicates that Lcb4p is bound to the cytoplasmic face of membranes.

To determine the nature of the association of Lcb4p-3HA with membranes, the P100 membrane fraction was treated with low pH (0.5 M potassium acetate, pH 4.5), with high salt (1 M NaCl) or with high pH (0.1 M sodium carbonate, pH 11), which release proteins that are weakly bound to membranes by ionic interactions, or with the chaotropic agent urea, which disrupts protein–protein interactions [22]. None of these treatments released Lcb4p-3HA from membranes (Fig. 5B), indicating that binding is quite strong. Treatment with 0.5 or 1.0% Triton X-100, which disrupts membranes, partially solubilized Lcb4p-3HA (Fig. 5C). Sonication of the samples to release protein trapped in multi-layered membrane vesicles, solubilized more but still not all of the protein (Fig. 5C). These results suggest that Lcb3p-3HA binds to a membrane protein which may be part of a large, detergent-resistant and sedimentable complex.

4. Discussion

A major finding of this work is that Lcb4p is found primarily in the Golgi, probably the *trans*-Golgi network, and in late endosomes/PVC (Figs. 2 and 3). Data (Fig. 2D) also show that Lcb4p cycles back and forth between the *trans*-Golgi network and late endosomes/PVC, like many other membrane-bound proteins (reviewed in [21]).

Until now there has been no indication of where DHS-P and PHS-P are synthesized in *S. cerevisiae* cells. Therefore, a key implication of our data is that they are made in the Golgi and in late endosomes/PVC. For synthesis to occur in these organelles some fraction of de novo synthesized DHS and PHS must escape acylation by the ceramide synthases in the ER and transit to the Golgi to be phosphorylated. Alternatively, breakdown of ceramide of complex sphingolipids could provide DHS or PHS, but there is no indication of such turnover.

Localization of Lcb4p to Golgi and late endosomes/PVC is unexpected because the biosynthetic enzymes for making DHS and PHS are located in the ER as are the Lcb3p and Ysr3p phosphatases and the Dpl1p lyase that degrade DHS-P and PHS-P. Because of this separation of biosynthetic and degradative pathways, DHS-P and PHS-P would need to be transported from the Golgi to the ER for breakdown to occur. It is known that exogenous LCBs must first be phosphorylated and then dephosphorylated in order for *S. cerevisiae* cells to efficiently incorporate them into ceramides [23–26]. Our data imply that phosphorylation of the majority of exogenous LCBs is catalyzed by Lcb4p on the Golgi and late endosomes/PVC followed by transit to the ER where they are dephosphorylated before incorporation into ceramides.

A general function for Lcb4p is to regulate LCBs and reduce their concentration back to the basal level following a heat-induced increase [27]. Our Lcb4p localization data extend this idea and argue that the rise in LCBs, which most likely occurs by de novo synthesis in the ER, would be followed by transport to the Golgi where their concentration would be reduced by phosphorylation. This prediction is supported by the rate of increase in the concentration of LCBs, which occurs 5–10 min prior to the increase in LCBPs [27]. If, as we hypothesize, LCBPs are made in the Golgi and late endosomes/PVC then they may function to regulate processes performed in these compartments including protein and lipid modification, processing, sorting, transport and degradation. Our analysis of the buoyant density of endosomes and Golgi suggest that Lcb4p or Lcb5p and LCBPs have a physiological function in one of these organelles. In RCD224 wild-type cells the Golgi and late endosomal marker proteins (Sec7p and Pep12p, respectively) coincided, whereas in RCD220 (*lcb4Δ lcb5Δ*) cells the Sec7p Golgi marker was shifted to a slightly lower buoyant density (Fig. 4). Further work will be needed to understand the physical basis for this change in buoyant density.

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