The Uracil Transporter Fur4p Associates with Lipid Rafts

Running title: Fur4p in lipid rafts

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†The abbreviations used are: Aureobasidin A, AbA; DHS, dihydrosphingosine; IPC, inositol-phosphoceramide; LCB, long chain base; LCBP, long chain base phosphate; MIPC, mannose-inositol-phosphoceramide (MIPC); (M(IP)2C, mannose-(inositol phosphate)2-ceramide; PHS, phytosphingosine
ABSTRACT

Sphingolipids are abundant components of eucaryotic membranes where they perform essential functions. To uncover new roles for sphingolipids we studied Saccharomyces cerevisiae lcb1-100 cells, which have a temperature-sensitive block in the first step in sphingolipid synthesis. We find that the level of all five species of the sphingoid long chain base intermediates is reduced 2- to 7-fold in cells grown at a permissive temperature and the level of complex sphingolipids is reduced 50%. In addition, lcb1-100 cells make no detectable phosphorylated sphingoid bases. Following transfer to a restrictive temperature (a heat shock), the level of the major sphingoid bases drops rather than transiently rising as in wild type cells. These changes affect lcb1-100 cells in multiple ways. Basal uracil transport by Fur4p is reduced 25% and, when cells are heat shocked, uracil transport activity falls rapidly and is not restored as it is in wild type cells. Restoration requires a functional secretory pathway and synthesis of complex sphingolipids, leading us to hypothesize that Fur4p associates with lipid rafts. The finding that Fur4p is insoluble in TritonX-100 at 4°C and behaves like a raft-associated protein on a density gradient supports this hypothesis. Raft association may be essential for regulating breakdown of Fur4p in response to stresses and other factors that govern uracil transport activity. Our results show that long chain bases do not contribute to the inactivation of Fur4p transport activity following heat stress, but they are essential for some later, but unknown, process that leads to degradation of the protein. Further studies using lcb1-100 cells should reveal new roles of sphingolipids in nutrient uptake and other membrane-dependent processes.
Sphingolipids are essential components of eucaryotic cells and are implicated in a growing number of cellular functions including signal transduction and association with sterols in membranes to form lipid rafts. To uncover new roles for sphingolipids we have studied Saccharomyces cerevisiae cells carrying a mutation that inhibits sphingolipid synthesis at a restrictive temperature. These studies demonstrate that delivery of the uracil transporter Fur4p to the plasma membrane requires synthesis of complex sphingolipids, which most likely reflects the need for de novo formation of lipid rafts, since we find that Fur4p is present in rafts.

Once viewed as experimental artifacts, the existence of lipid rafts is now generally accepted because they have been identified as important elements in many cellular functions including trafficking and sorting of membrane proteins and lipids (reviewed in 1,2). More recently lipid rafts have been recognized as platforms for the organization and regulation of signal transduction cascades (reviewed in 3,4), as critical components for activating the mammalian immune system (reviewed in 4-6), and as a key element in regulating the cellular level of cholesterol (reviewed in 7,8).

Lipid rafts in S. cerevisiae contain ergosterol and complex sphingolipids (9,10) whereas the rafts in higher eucaryotes contain cholesterol and sphingomyelin or glycosphingolipids (1). The complex sphingolipids in S. cerevisiae are inositol-phosphoceramide (IPC¹), mannose-inositol-phosphoceramide (MIPC) and mannose-(inositol phosphate)₂-ceramide (M(IP)₂C (11). Sphingolipid synthesis up through formation of ceramide occurs in the endoplasmic reticulum and then ceramide is transported to the Golgi where the polar head groups are added (11,12). Lipid raft formation occurs primarily in the Golgi apparatus in mammals (13), but the situation is different in yeast. At least for
yeast proteins with a glycosphatidylinositol-anchor, current evidence suggests that they associate with lipid rafts in the endoplasmic reticulum (10): presumably such rafts contain ceramides as their sphingolipid component. Other studies on Pma1p, a proton transporter and the most abundant protein in the plasma membrane of *S. cerevisiae* cells (14), argue that raft association occurs in the Golgi apparatus (15), where IPC, MIPC and M(IP)₂C would be expected to be the sphingolipid component of rafts or, alternatively, association occurs in the endoplasmic reticulum (16).

Our interest in lipid rafts arose from studies on *S. cerevisiae* cells carrying the *lcb1-100* mutation, first identified as *end8-1* because cells are blocked in endocytosis when shifted to a restrictive temperature (37°C) (17). *LCB1* encodes a subunit of serine palmitoyltransferase (18) the enzyme catalyzing the first committed step in sphingolipid synthesis in which serine is condensed with palmitoyl-CoA to yield the long chain base 3-ketodihydrosphingosine (reviewed in 11). A serine palmitoyltransferase molecule containing an Lcb1-100 subunit is presumably inactivated when cells are shifted to 37°C. The exact level of enzyme activity cannot be determined because cell-free extracts have no measurable activity even when prepared from cells grown at 25°C (19). Nonetheless, *lcb1-100* cells are useful because sphingolipid synthesis is reduced rapidly upon shifting to 37°C (20) and any process dependent upon sphingolipid intermediates or complex sphingolipids will be impaired.

Even at a permissive temperature *lcb1-100* cells grow slowly on complex medium and barely grow on defined medium unless it is supplemented with yeast extract or casamino acids, suggesting that nutrient uptake is impaired because of reduced amounts of sphingolipids. In the experiments reported here we focus on uracil uptake by the Fur4
protein, the only uracil transporter in \textit{S. cerevisiae} cells, which has been studied extensively. Fur4p synthesis begins in the endoplasmic reticulum where its twelve transmembrane domains are inserted into the membrane (21). During transit through the secretory pathway it is phosphorylated but is otherwise unmodified upon reaching the plasma membrane (22). The amount of Fur4p in the plasma membrane is highly regulated and the protein is degraded in response to stresses including heat stress and high concentrations of uracil in the culture medium (22-24). Degradation involves a series of steps including phosphorylation, ubiquitination, endocytosis and transport to the vacuole where the protein is hydrolyzed (25-27).

As predicted, we find that the steady-state level of the sphingolipid biosynthetic intermediates dihydrosphingosine (DHS) and phytosphingosine (PHS) are very low in \textit{lcb1-100} cells grown at a permissive temperature and complex sphingolipids are reduced 50%. When cells are switched to a restrictive temperature, DHS and PHS do not transiently increase like they do in wild type cells, but instead drop to a low level, which explains why complex sphingolipid synthesis stops. We find that these changes in sphingolipids impact uracil transport. For example, uracil transport activity is reduced even in cells grown at the permissive temperature and, following a shift to a restrictive temperature (a heat shock), activity goes down and is not restored whereas in wild type cells transport activity is restored within two hours. To explain these results, we hypothesized that the block in sphingolipid synthesis prevents raft formation, that Fur4p is present in lipid rafts, and that rafts are essential for delivery of newly synthesized Fur4p to the plasma membrane. Our data support these hypotheses. Furthermore, our data establish that long chain bases are
not required for inactivation of Fur4p uracil uptake activity, but are required for a later step in Fur4p breakdown.

**MATERIALS AND METHODS**

*Strains, plasmids, and media*— Strains used in these experiments are listed in Table 1. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), YPAUD (YPD plus 20 µg/ml of adenine sulfate and uracil), complete synthetic medium (SD: 0.34% yeast nitrogen base (Difco), 1% ammonium sulfate, 2% glucose, 30 mg/l of adenine and tyrosine, and 20 mg/l each of histidine, leucine, lysine, methionine, uracil, and tryptophan) or synthetic media lacking uracil (SD-Ura) to select cells transformed with a plasmid carrying *URA3*. Solid media contained 2% agar. Casamino acids (Difco) were added to a final concentration of 0.5% to SD medium. Unless otherwise indicated, transformed cells were grown at 30°C on SD-Ura plates, pooled using deionized water, diluted into YPD or YPAUD medium and grown overnight at 25°C. In some experiments Fur4p was overproduced using cells transformed with YEp352fF (2µm, *URA3, FUR4*, 28), 195gF (2µm, *URA3, GAL10-FUR4*, 23) or pFL38gG-GFP (*CEN, URA3, GAL10-FUR4::GFP*, 29).

*Lipid analysis*— Sphingoid long chain bases (LCBs) and their phosphorylated species (LCBPs) were extracted from yeast cells, coupled to 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and the derivatized compounds were analyzed by high performance liquid chromatography (30).

Complex sphingolipids were measured by radiolabeling cells with [2-³H]-myo-inositol (American Radiochemicals Inc. #116, 20 Ci/ml) as described previously except that cells were grown for 16-18 hr in the presence of the radioisotope to an A600 of 1 and
then lipids were extracted, deacylated and chromatographed on thin layer plates (31). Radioactivity was detected by using a BioScan apparatus and sphingolipid standards, co-chromatographed with each radiolabeled sample, were detected after spraying the thin-layer plate with 10% copper sulfate in 8% phosphoric acid, followed by charring at 160°C. Radiolabeled sphingolipids were quantified by chromatography on EDTA-treated silica gel-impregnated paper (Whatman SG81) and analyzed as described previously (31).

**Uracil uptake assay** — The assay was based upon published procedures showing that uptake of uracil requires Fur4p (22,23). Cells were grown in YPD medium overnight at 25°C to an A_{600} of 0.3-0.4 and 3 A_{600} units were harvested by centrifugation at 1900 x g for 3 minutes at room temperature. Cells were suspended in 1 ml YPD broth pre-warmed to 30°C, and 56 nCi of [^{14}C]-Uracil (57 mCi/m mole, Sigma) was added. After 10 minutes of incubation at 30°C, the cells were filtered onto a Whatman GF/C filter and washed 3 times with 2 ml of ice-cold water. Filters were dried, suspended in 4 ml of Ultima Gold XR scintillation fluid (Packard; Meriden, CT), and radioactivity was measured in a liquid scintillation counter. Uptake was linear over 10 min and required Fur4p (data not shown).

**Secretion of proteins into the culture medium** — This procedure is similar to a published procedure (32). Cells were grown overnight in YPAUD at 25°C to an A_{600} of 0.2-0.3 and 1.5 A_{600} units were harvested by centrifugation for 3 minutes at 1,900 x g at room temperature. The cell pellet was washed once with 1 ml of SD lacking methionine and cysteine, then suspended in 350 µl of the same medium containing 150 µCi of Trans[^{35}S]-label (ICN Biomedicals, Inc.), 0.06 mg/ml BSA, and 1 mM phenylmethylsulfonyl fluoride. The reaction was incubated at 25°C for 20 minutes or 37°C for 15 minutes and stopped by a 5 second centrifugation in a microcentrifuge to yield cell pellet and medium fractions.
The medium fraction (300 µl) was transferred to a new tube containing 20 µl of both 200 mM NaN₃ and 200 mM NaF and centrifuged for 1 minute at 4°C to remove any remaining cells. Proteins were precipitated from the medium fraction by a 5 min incubation on ice in the presence of TCA (final concentration 6.5%) and deoxycholic acid (final concentration 0.6 mg/ml) and collected by centrifugation for 10 minutes at 4°C. The precipitate was washed twice with 1 ml acetone (-20°C), air dried at room temperature, and dissolved in 30 µl of sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.1% Bromphenol Blue, and 100 mM dithiothreitol). The cell pellet was suspended in 300 µl of sample buffer and vortexed with glass beads (½ volume) for 4 minutes at room temperature. Whole cells and glass beads were removed by centrifugation for 5 seconds in a microfuge. The cell-free lysate and the TCA-precipitated proteins were treated for 5 minutes in a 100°C boiling water bath and resolved by SDS-PAGE (8% for the media fraction and 10% for the cell pellet fraction). Radioactivity was quantified by using a Molecular Dynamics Storm Phosphorimager.

Isolation of lipid rafts — Lipid rafts were isolated essentially as described previously (10). Cells were grown overnight in YPD at 25°C to an \( A_{600nm} \) of 0.3-0.5, harvested by centrifugation, and washed once with 1 ml of \( H_2O \). The cell pellet was suspended in 350 µl of TNE buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA) plus protease inhibitors (final concentrations of 0.2 mM 4-[2-aminoethyl] benzenesulfonyl fluoride hydrochloride, 3 µM E-64, 4 µM Pepstatin A, 1 mM 1,10-Phenanthroline (Calbiochem), and broken by vortexing with one-half volume of glass beads for 8 cycles (30 sec of vortexing followed by 30 sec on ice). Unbroken cells and beads were removed by centrifugation for 5 minutes at 500 x g. The chilled supernatant fluid (250 µl) was incubated with Triton X-100 (1% final
concentration) for 30 min on ice followed by addition of Optiprep (Nycomed, Oslo, Norway) to give a final concentration of 40% (w/v). The sample was placed in a centrifuge tube and overlaid with 1.2 ml 30% Optiprep in TXNE (TNE + 0.1% TritonX-100) followed by 200 µl of TXNE, and centrifuged for 2 hr at 55,000 rpm in a Beckman TLS55 rotor at 4º C. Six equal fractions, collected starting from the top of the gradient, were diluted to 1 ml with H₂O and proteins were precipitated with TCA (final concentration 10%) for 30 minutes on ice. Precipitated proteins were collected by centrifugation in a microfuge for 20 minutes at 4ºC and the pellet was dissolved in 15 µl of 1 M Tris base, and 35 µl of dissociation buffer (0.1 M Tris-Cl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.02% Bromphenol Blue). Samples were heated at 37ºC for 10 min, resolved on 10% SDS-PAGE using a Tricine buffer system (33) and proteins were detected by immunoblotting.

**Immunoblots** — Immunoblotting was performed by transferring proteins separated on SDS-PAGE to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) followed by blocking overnight in 2% powdered skim milk made in TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Primary antibodies were rabbit anti-Fur4p (1/40,000 dilution; Dr. R. Haguennauer-Tsapis), rabbit anti-Pma1p (1/50,000 dilution; Dr. R. Serrano or A. Chang), and mouse anti-Pgk1p (1/500 dilution; Molecular Probes). Secondary antibodies were polyclonal Rabbit IgG (1/20,000 dilution; Molecular Probes) and monoclonal Mouse IgG (1/3,000 dilution; Molecular Probes). Bound antibodies were detected using ECF substrate (Amersham Pharmacia, Buckinghamshire, England) and the fluorescent signal was quantified by using a Molecular Dynamics Storm PhosphoImager equipped with ImageQuant software.
Immunoprecipitation — TCA precipitated proteins were first neutralized with 20 µl of Tris base then dissolved in 30 µl of the same dissociation buffer as used for isolating lipid rafts except that it lacked 2-mercaptoethanol. Samples were heated for 10 minutes at 37ºC, and chilled on ice. TNET buffer (0.6 ml; 50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-00) was added, and insoluble material was removed by centrifuging at top speed for 30 minutes in a microcentrifuge at 4ºC. The supernatant fluid (200µl plus 400µl of TNET) was incubated with 40 µl of protein A-sepharose beads (10% w/v in TNET) with gentle agitation. The beads were removed by centrifuging for 3 minutes at 3,000 rpm at 4ºC, and proteins were immunoprecipitated from the supernatant fluid by adding beads as mentioned above and 4 µl of anti-GFP antibody (Torrey Pines Biolabs, San Diego, CA). IP reactions were incubated overnight at 4ºC with gentle agitation, and beads were collected as described above and were washed 4 times with 0.5 ml of TNET buffer. Proteins were eluted from the beads with 30 µl of dissociation buffer and subjected to heat treatment at 37ºC for 10 minutes. Proteins were resolved by SDS-PAGE (10%) in the tricine buffer system; the gels were dried and exposed on a Kodak phosphor storage screen. Radioactive proteins were analyzed by using a Phosphorimager.

RESULTS

The level of sphingolipid intermediates and complex sphingolipids is reduced in lcb1-100 cells — The extreme instability and temperature sensitivity of serine palmitoyltransferase activity and the slow growth phenotype of lcb1-100 cells (see below) suggested that they have a reduced capacity, even at a permissive temperature, to make
sphingolipid biosynthetic intermediates including the long chain bases (LCBs) DHS and PHS. Since heat induces a transient increase in LCBs in wild type cells (34-36) it seemed likely that the thermoinstability of serine palmitoyltransferase in \textit{lcb1-100} cells would prevent the transient increase. This increase regulates the transient cell cycle arrest, in an unknown manner, that occurs shortly after cells are heat shocked (19) and also mediates endocytosis and restoration of the actin cytoskeleton by an unknown mechanism (20,37).

In addition, we examined LCB phosphates (LCBPs), which have not been examined previously in \textit{lcb1-100} cells. LCBPs also transiently increase during heat shock (36,38) and are thought to mediate some processes that protect cells against heat stress (reviewed in 11).

LCBs and LCBPs were quantified by tagging them with a fluorescent reagent after extraction from cells followed by HPLC (30). Parental RH1800 (\textit{LCB1}) cells behaved as expected and all species of LCBs increased transiently shortly after cells were shifted from 25°C to 37°C (Fig. 1A; maximum fold increase from time 0: C\textsubscript{16}-PHS, 1.3-fold; C\textsubscript{18}-DHS, 2.5-fold; C\textsubscript{18}-PHS, 4-fold; C\textsubscript{20}-DHS, greater than 30-fold; C\textsubscript{20}-PHS, 13-fold). RH3809 (\textit{lcb1-100}) cells behaved very differently. Even at 25°C the basal level LCBs was reduced from 2 to 7-fold compared to parental cells (Fig. 1B). These data differ from previous data which indicated that \textit{lcb1-100} cells grown at 25°C contain a normal level of LCBs and that the level did not change during heat shock (19). Second, the major species of LCBs in \textit{lcb1-100} cells, C\textsubscript{16}-DHS, C\textsubscript{18}-DHS and C\textsubscript{18}-PHS, did not increase after heat treatment. Instead they steadily decreased and reached a plateau after about 30 min. C\textsubscript{20}-PHS did increase to about 1 pMole/A600 units of cells, but this concentration is still only ten percent of the level found in parental RH1800 cells and the level stayed elevated in mutant cells.
rather than dropping as it did in parental cells. Finally, RH3809 cells did not make any detectable C$_{20}$-DHS at any time point.

LCBPs also transiently increase during heat shock and this is what we observe for RH1800 (wild type $LCB1$) cells where all species of LCBPs transiently increase, although each species shows different kinetics (Fig. 1C). Surprisingly, LCBPs were not detected in $lcb1-100$ (RH3809) cells with the limit of detection being 0.1 pmole/A600 unit of cells (data not shown).

The reduced basal level of LCBs in $lcb1-100$ cells could cause a reduction in the concentration of IPC, MIPC and M(IP)$_2$C. To examine this possibility, sphingolipids were measured by growing cells for at least ten generations at 25°C in the presence of [$_3^H$]-inositol. Cells were maintained in log phase growth during the labeling period to avoid the possibility that they would differentially take up radiolabel upon entry into stationary phase. Lipids were extracted, deacylated to remove glycerophospholipids and radioactive sphingolipids were analyzed in two ways. First, they were chromatographed on a high performance thin-layer plate and radioactive species were localized by using a Bioscan apparatus. This approach gives good resolution of IPC, MIPC and M(IP)$_2$C, and was used to determine which species were present and what their relative level was. However, quenching within each band impairs quantification. To avoid quenching, samples were also chromatographed on SG81 paper and the chromatogram was cut into sections and analyzed in a liquid scintillation spectrometer. This approach does not separate IPC from MIPC.

We observed that IPC plus MIPC were reduced 77% in $lcb1-100$ cells and that M(IP)$_2$C was reduced 22% compared to parental RH1800 cells (Table 2). The total
reduction in sphingolipid content is about 50%. This value is based upon the fact that M(IP)_2C has twice the number of radioactive inositol groups as do IPC and MIPC. \[^{3}\text{H}\]-Glycero-P-inositol, the deacylation product of phosphatidylinositol, served as an internal control for radiolabeling. It was present at the same level in both strains, showing that the reduced radiolabeling of M(IP)_2C and IPC plus MIPC is due to a reduced level of these compounds. From analysis of the thin-layer chromatogram is was clear that the MIPC band was reduced in \textit{lcb1-100} cells and so was the band corresponding to IPC with a dihydroxy-fatty acid (data not shown). We conclude that the total concentration of complex sphingolipids is reduced about 50% in unstressed \textit{lcb1-100} cells relative to wild type cells.

Because the uptake of \[^{3}\text{H}\]-inositol drops when \textit{lcb1-100} cells are heat shocked (data not shown) it was not possible to measure sphingolipids after heat shock using a radiolabeling procedure. However, because LCB levels drop in \textit{lcb1-100} cells following heat shock (Fig. 1B), it seems likely that sphingolipid synthesis also drops and this would further impair any cellular process dependent upon continued synthesis of LCBs, ceramide or complex sphingolipids.

\textit{The protein encoded by the \textit{lcb1-100} allele has Ala381 to changed to Thr} — To determine the location of the \textit{lcb1-100} mutation we transformed RH3809 cells with a series of overlapping DNA fragments and looked for complementation of the mutant allele by growing cells at 37°C. A DNA fragment spanning bases 900 to 1350 of \textit{LCB1} gave rise to cells that grew at 37°C. This region of the \textit{lcb1-100} chromosomal locus was recovered by gap repair of a plasmid carrying \textit{LCB1} and the DNA sequence of both strands of several gap-repaired plasmids was determined. Nucleotide 1141 was found to be mutated from G to A resulting in Ala-381 being changed to Thr. Supporting the idea that this mutation
inactivates the gene is the finding that gap-repaired plasmids carrying the mutation did not restore grow at 37°C when transformed into RH3809 cells whereas the wild type \textit{LCB1} allele did restored growth (data not shown). Using the proposed 3-dimensional model for serine palmitoyltransferase (39), Ala-381 is predicted to be at the interface between the Lcb1 and Lcb2 subunits, in the vicinity of the predicted active site. Thus the Ala-381 to Thr change may affect catalysis, interaction of the two subunits or both.

\textit{Amino acid and uracil uptake are impaired in lcb1-100 cells} — We observed that cells carrying the \textit{lcb1-100} allele grew slower at permissive temperatures than did cells with the wild type \textit{LCB1} allele. For example, strains with the \textit{lcb1-100} allele (RH3809 and RH3804) grew more slowly than parental RH1800 cells on YPD plates (Fig. 2) and barely grew on defined medium (SD, Fig. 2) suggesting impaired nutrient uptake. This hypothesis is supported by the observation that adding 0.5% casamino acids to SD plates improves growth of \textit{lcb1-100} cells (SD + CA, Fig. 2).

We also found that uracil uptake was abnormal and focused on it because there is only one uracil transporter in \textit{S. cerevisiae} cells, Fur4p, and much is known about the regulation of uracil uptake activity (29). RH3809 (\textit{lcb1-100}) cells grown at the permissive temperature of 25°C had 25\% less uracil uptake activity as did wild type RH1800 cells (Fig. 3, zero min time point). Upon shifting to 37°C, uracil uptake activity decreased within 30 min in both strains. These results are similar to published data for wild type cells subjected to various stresses where it is known that Fur4p is degraded (23). Within the next 30 min the activity began to rise in wild type cells and it returned to about 65\% of the starting activity by 150 min. In contrast, mutant RH3809 cells did not restore uptake activity.
Nearly all published studies of Fur4p have used cells overproducing the protein some 20 to 40-fold above the endogenous level so that the protein level could be measured by immunoblotting (29) and references therein. Therefore, we examined lcb1-100 cells carrying FUR4 on a multicopy plasmid and found that uracil transport activity behaved the same as it did in cells with only chromosomal FUR4 (data not shown).

Restoration of uracil uptake activity after heat shock requires exocytosis — To begin to understand why uracil uptake activity is not restored in lcb1-100 cells after heat shock, we first determined if restoration requires the secretory pathway. For these experiments the restoration of uptake activity after heat shock was compared in wild type and sec6 mutant cells which have a temperature-sensitive block in exocytosis. Sec6p is part of the exocyst complex that is necessary for transport of proteins (40) and sphingolipids (41) from the Golgi to the plasma membrane. We found that sec6 cells fail to restore uracil uptake activity after heat shock (Fig. 4), indicating that restoration requires a functional secretory pathway.

Restoration of uracil uptake activity after heat shock requires sphingolipid synthesis — Next we determined if uracil uptake activity could be restored in lcb1-100 cells if they were allowed to make sphingolipids at the restrictive temperature. Sphingolipid synthesis can be restored in lcb1-defective strains by adding PHS to the culture medium (18). We found that addition of 5 μM PHS to the culture medium 15 min prior to heat shock restored uracil uptake activity in lcb1-100 cells (Fig. 5) with the same kinetics and degree of restoration as was seen in wild type cells (Fig. 3). In contrast, activity in the control cells treated with ethanol, the carrier for PHS, was not restored (Fig. 5).
Exogenous PHS could restore uracil uptake activity by acting as a signaling molecule to regulate some step in exocytosis as has been suggested (32,42) or it could act by restoring sphingolipid synthesis in lcb1-100 cells. To determine which of these possibilities is correct, lcb1-100 cells were treated with PHS and with Aureobasidin A (AbA) which blocks sphingolipid synthesis by inhibiting IPC synthase, the enzyme that transfers inositol phosphate onto ceramide to form IPC (43). Cells were grown in the presence of increasing concentrations of AbA to find the lowest concentration that inhibited growth under the culture conditions used (data not shown). Cells treated with PHS and AbA behaved like the ethanol-treated cells and did not restored uracil uptake (Fig. 5). We conclude that restoration of uracil uptake activity in heat shocked lcb1-100 cells requires de novo synthesis of one or more of the complex sphingolipids, IPC, MIPC and M(IP)2C.

*Exocytosis is selectively blocked in heat shocked lcb1-100 cells* — We next determined whether exocytosis is completely or selectively blocked in lcb1-100 cells following heat shock by examining secretion of proteins into the culture medium. Cells were pulse labeled with [35S]-amino acids prior to heat shock and radioactive proteins excreted into the culture medium were examined at the start (0 min) and after 60 and 120 min of heat shock. At 0 min the pattern of radioactive proteins secreted by RH3809 cells is similar but not identical to wild type RH1800 cells (Fig. 6). The primary difference is a higher concentration of the 120 and 173 kDa bands in the lcb1-100 cell samples. At the 60 min time point secretion of all proteins except the 173 kDa protein is reduced in mutant RH3809 cells compared to wild type RH1800 cells. At the 120 min time point secretion of proteins into the culture medium is mostly restored in RH3809 cells with the major missing bands being those of 49 and 111 kDa. As a control to measure the rate of protein
synthesis, radioactivity in the pellet fractions was quantified by SDS-PAGE and phosphorimage analysis, which was used to sum all pixels in a lane. At the 0 min time point \textit{lcb1-100} cells incorporated 50\% as much radioisotope into proteins as did the wild type cells while at 60 and 120 minutes the value was 37\% and 32\%, respectively (data not shown). Even without correcting for reduced total protein synthesis, it is apparent that secretion of some but not all proteins is blocked after \textit{lcb1-100} cells are heat shocked. We conclude from these data that there is a selective, not a complete block in the secretory pathway when \textit{lcb1-100} cells are heat shocked.

\textit{Fur4p is present in detergent insoluble complexes} — It has recently been shown that some membrane proteins in yeast including Gas1p and Pma1p are found in detergent insoluble complexes or lipid rafts, experimentally identified by insolubility in 1\% Triton X-100 at 4°C and by their low buoyant density in a Optiprep density gradient (10). Raft association and transport of Pma1p to the plasma membrane depend upon sphingolipid synthesis (15). Thus, one explanation for the failure of \textit{lcb1-100} cells to restore uracil uptake activity is that \textit{de novo} sphingolipid synthesis ceases after heat shock thereby preventing new rafts from forming in the Golgi which blocks transit of raft-associated proteins to the plasma membrane.

To test this hypothesis we first determined if Fur4p is present in lipid rafts. Wild type RH1800 and \textit{lcb1-100} (RH3809) cells were disrupted, extracted at 4°C with 1\% Triton X-100 and the extracts were fractionated by centrifugation on an Optiprep density gradient. The raft-associated control protein Pma1p localized to the top of the gradient primarily in fractions 1 and 2, in both types of cells grown either at 25°C or grown for 120 min at 37°C (Fig. 7A). Fur4p also localized in fractions 1 and 2, indicating that it associates with lipid
rafts. Phosphoglycerokinase was used as a control for a soluble, non-raft protein. It localized in fractions 4, 5 and 6 at the bottom of the gradient. To verify that we were dealing with detergent insoluble complexes, the Triton X-100 extractions were performed at 4°C and at 30°C. Pma1p and Fur4p were only insoluble when the extraction was performed at 4°C (data not shown), indicating that the proteins were partitioning into the detergent insoluble fraction. In this particular experiment (Fig. 7A) there appears to be staining by the Fur4p antibodies in fractions 4-6 of the RH3809 37°C sample. This staining was non-specific because it did not appear in other experiments, and most importantly, it was not present when Fur4p was overproduced (Fig. 7B).

After 2 hrs at 37°C the concentration of Fur4p in wild type RH1800 cells is less than in the 25°C sample taken before heat shock (Fig. 7A, top immunoblot fractions 1-3, compare 25°C and 37°C). This reduction in Fur4p is reflected in reduced uracil transport activity after 2 hrs of heat shock (Fig. 3). These results are in agreement with previous studies showing that Fur4p is rapidly degraded and uracil transport activity lost in wild type cells following heat shock, but that both are restored by 2 hrs (22,23). The situation in lcb1-100 cells (RH3809) appears to be similar, but is actually very different. The immunoblot shown in Fig. 7A indicates that there is slightly less Fur4p in raft fractions 1 and 2 in the cells grown 2 hrs at 37°C compared to the 25°C sample. However, the Fur4p present in fractions 1 and 2 of the 37°C sample is not newly synthesized protein as it is in the wild type cells, but instead represents protein that failed to be degraded. This explanation is based upon previous reports that Fur4p fails to be degraded when lcb1-100 cells are heat shocked (44). Therefore, the uracil transport data (Fig. 3) combined with the immunoblot data (Fig. 7A) show for the first time that Fur4p transport activity is inactivated.
by heat stress in *lcb1-100* cells and that, whatever the inactivation process is, it does not require a transient increase in long chain bases.

Nearly all published studies of Fur4p have used cells overproducing the protein, so we determined if the overproduced protein associated with rafts. Indeed, most of the Fur4p in wild type RH1800 or mutant RH3804 cells transformed with a 2 micron vector carrying *FUR4* under control of its own promoter (YEp352fF) localized to the top two fractions of an Optiprep gradient just like the raft-bound Pma1 control protein (Fig. 7B). We conclude from these results that Fur4p associates with lipid rafts.

Association of Fur4p with lipid rafts provides an explanation for why *lcb1-100* cells do not restore uracil transport activity after heat shock. Heat shock would cause DHS and PHS levels to drop (Fig. 2) and this change would set off a chain reaction so that ceramide synthesis in the endoplasmic reticulum and complex sphingolipid synthesis in the Golgi would be greatly reduced. As a consequence, raft formation either in the endoplasmic reticulum or Golgi would be impaired, as would raft-dependent processes including transport of newly synthesized Fur4p to the plasma membrane.

We attempted to garner further support for this hypothesis by measuring the rate at which *de novo* synthesized Fur4p associates with rafts. Because the concentration of endogenous Fur4p is low, we used the galactose-inducible *GAL1* promoter to drive overexpression as described previously (22). In addition, we used a *FUR4* allele with *GFP* fused to the C-terminus (27) so that the protein could be immunoprecipitated using a commercially available antibody. Anti-Fur4p is not commercially available and our stock was insufficient for immunoprecipitation experiments. Unfortunately, the *lcb1-100* strains used in our experiments only grow well with glucose as the carbon source and this
prevented induction of \(GAL\) gene expression. To overcome this impasse we switched to the W303 strain background. Using established procedures (15,22) to pulse label proteins with \([^{35}\text{S}]\)-amino acids, we obtained measurable amounts of immunoprecipitated, radiolabeled Fur4-GFPp prior to isolation of lipid rafts. However, following raft isolation by treatment of the sample with 1% Triton X-100, immunoprecipitable, radioactive Fur4-GFPp was undetectable (data not shown). Thus, for these technical reasons it was not possible to directly measure the rate at which \textit{de novo} synthesized Fur4p associates with lipid rafts.

**DISCUSSION**

Our results demonstrate that sphingolipid synthesis is essential for delivery of Fur4p to the plasma membrane (Fig. 5) and that the protein is present in lipid rafts (Fig. 7). The sphingolipid requirement for delivery to the plasma membrane most likely reflects the need for Fur4p to associate with lipid rafts as they form in the secretory pathway. We also find that the steady-state level of complex sphingolipids in \textit{lcb1-100} cells is 50% lower than in wild type cells (Table 2). This reduction may be the reason why \textit{lcb1-100} cells grow poorly on a defined medium unless it is supplemented with amino acids (Fig. 2) and why the cells have only about 75% of the wild type level of uracil transport activity at permissive temperatures (Fig. 3). Finally, our results establish that long chain bases are not essential for the process of inactivating Fur4p uracil transport activity, but that they are necessary for a later step in Fur4p breakdown.

Since lipid raft formation requires complex sphingolipids, we reasoned that the failure of \textit{lcb1-100} cells to restore uracil transport activity after heat shock was due to a
block in raft formation. This hypothesis predicts that Fur4p should be associated with lipid rafts and this is what we found experimentally (Fig. 7). Another prediction of this hypothesis is that delivery of most proteins to the plasma membrane or secretion of proteins into the culture medium should be unaffected in heat shocked lcb1-100 cells, since many proteins are not associated with lipid rafts and their transport to the plasma membrane should be normal. Indeed, we observed that secretion of 5 out of 7 radioactive proteins into the culture medium was restored in lcb1-100 cells 120 min after heat shock (Fig. 6). Cells with a complete block in the secretory pathway accumulate secretory vesicles or enlarge their secretory compartments, which can be observed by electron microscopy (45). We analyzed lcb1-100 cells before and after heat shock by electron microscopy and found no differences compared to type cells (data not shown). Together these data strongly support the hypothesis that restoration of uracil transport activity requires de novo raft formation, which in turn requires de novo sphingolipid synthesis.

The reported lability of serine palmitoyltransferase activity and other phenotypes of lcb1-100 cells suggested that they do not make normal levels of LCBs. Quantification of LCBs in lcb1-100 cells showed that their basal value was reduced from 2 to 7-fold compared to parental cells even when cells were grown at the permissive temperature of 25°C (Fig. 1). We also found that lcb1-100 cells had no detectable basal level of LCBPs nor did heat shock increase LCBPs. The absence of LCBPs may occur because the concentration of LCBs in lcb1-100 cells is below the Km for the two lipid kinases, Lcb4p and Lcb5p, that phosphorylate LCBs to yield LCBPs. Specific functions for LCBPs have not been identified in S. cerevisiae. lcb4Δ lcb5Δ cells, which lack all detectable LCBPs, grow normally and only show a very slight reduction in resisting heat stress (36) and
references therein). So the lack of LCBPs in *lcb1-100* cells seems unlikely to be the cause of the reported phenotypes: the most likely cause is the reduction in LCBs (Fig. 1) and complex sphingolipids (Table 2).

Our observation that *lcb1-100* cells have a reduced basal level of LCBs and that the levels drop rapidly after cells are heat shocked at 37°C, except for C20-PHS (Fig. 1), provide a chemical basis for explaining why signal transduction pathways regulated by LCBs are disrupted when *lcb1-100* cells are heat shocked. DHS and PHS have been shown to regulate the protein kinases Pkh1p and Pkh2p (46–48), which in turn regulate the kinase activity of Pkc1p and probably Ypk1p and Ypk2p that control endocytosis (47,48), actin cytoskeletal restoration following heat shock (47) and the cell integrity pathway (49) in ways that are not entirely understood. In contrast to our data, it was previously reported (19) that the basal level of LCBs in *lcb1-100* cells was similar to wild type and dropped very little upon heat shock. The different results are probably not due to strain differences since their strains and ours originated in Riezman’s laboratory and carry the *lcb1-100* allele. Perhaps the differences are due to the way in which lipids are extracted and processed before HPLC analysis.

The earliest known event in stress-induced degradation of Fur4p is phosphorylation of serine residues within a PEST sequence mediated in part by the casein kinases Yck1p and Yck2p (50). Subsequently, the protein is ubiquitinated, internalized by endocytosis and degraded in the vacuole (27,28). Heat shocked *lcb1-100* cells do not degrade overproduced Fur4p unless given exogenous PHS (44). Presumably a transient increase in PHS is needed to facilitate some step in the degradation pathway, but the exact function of PHS is unknown. Our data (Fig. 7), obtained by measuring endogenous, not
overproduced, Fur4p, confirm this conclusion. In addition, our data show that uracil uptake activity declines rapidly in lcb1-100 cells following a heat shock, leading to the important conclusion that PHS (or any other LCB or LCBP) does not regulate the inactivation of Fur4p uptake activity. The molecular nature of inactivation is unknown. Future studies will need to address this question and determine if PHS regulates phosphorylation, ubiquitination, protein internalization or some subsequent event in the degradation process.

It should now be possible to identify the regions of Fur4p necessary for raft association and whether or not such association if essential for uracil transport activity. Rafts are thought to be important centers for integrating and modulating signal transduction pathways (3-5) and they may thus be critical for regulating breakdown of Fur4p in response to stresses and other factors that govern uracil transport activity.

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Figure Legends

Fig. 1. **Changes in LCBs and LCBPs during heat shock.** RH1800 (*LCB1*) and RH3809 (*lcb1-100*) cells were grown to an A600 nm of 0.3 to 0.4 in YPD medium at 25°C and at time zero the cultures were shifted to 37°C. LCBs and LCBPs were extracted, derivatized with 6-aminoquinolyl-N-hydroxysuccinimidy carbamate and analyzed by HPLC. Values are the average for duplicate samples ± standard deviations. The LCB values in pmoles/A600 units at the zero time point for RH1800 cells were: C16 DHS = 8.9, C18 PHS = 67, C18 DHS = 20, C20 PHS = 1.6 and C20 DHS = <0.1. The LCB values at the zero time point for RH3809 cells are: C16 DHS = 3.4, C18 PHS = 10.3, C18 DHS = 7.0, C20 PHS = <0.1 and C20 DHS = <0.1. The LCBP values at the zero time point for RH1800 cells were: C16 DHSP = 4.4, C18 PHSP = 5.9, C18 DHS = 1.3, C20 PHS = <0.1 and C20 DHS = <0.1.

Fig. 2. **Nutrient uptake limits the growth of lcb1-100 cells.** Strains RH1800 (wild type), RH3804 (*lcb1-100*) and RH3809 (*lcb1-100*) were streaked onto to agar plates containing YPD, SD or SD supplemented with 0.5% casamino acids (SD+CA) and incubated at 30°C for 4 days.

Fig. 3. **Uracil uptake activity is not restored in lcb1-100 cells after heat shock.** Cells were grown overnight at 25°C to early log phase, the 0 min sample was harvested, and then cultures were shifted to 37°C. Values represent the average of 2 cultures assayed in duplicate (n = 4) ± the standard deviation (* indicates p = 0.012 and ** indicates p < 0.001 determined by using the Student’s t-test).
Fig. 4. **Restoration of uracil uptake activity after heat shock requires protein secretion.** Uracil uptake activity was compared in wild type (NY13) and sec6-4 (NY17) cells after transfer of cultures from 25°C to 37°C. Values represent the average of 2 cultures assayed in duplicate (n = 4) ± the standard deviation (* indicates $p < 0.00001$ determined by using the Student’s $t$-test).

Fig. 5. **Restoration of uracil uptake activity in heat shocked lcb1-100 cells requires synthesis of complex sphingolipids.** Uracil uptake activity was compared in lcb1-100 cells (RH3809) treated with PHS, treated only with vehicle (EtOH), or treated with PHS and aureobasidin A (PHS + AbA), which blocks conversion of ceramide to IPC, MIPC and M(IP)2C. PHS and AbA stock solutions were prepared in 95% EtOH and diluted into cultures to give final concentrations of 5 µM PHS, 1 µg/ml AbA and 1% EtOH. Treatments were initiated 15 minutes before transfer of cultures from 25°C to 37°C. Values represent the average from 4 culture assayed in duplicate (n = 8) ± the standard deviation (* indicates $p < 0.00001$ determined by using the Student’s $t$-test).

Fig. 6. **Secretion of proteins into the culture medium is selectively blocked in lcb1-100 cells following heat shock.** Radioactive proteins secreted by RH1800 and RH3809 cells into the culture medium were measured prior to heat shock (0 min) and after 60 and 120 min of heat treatment at 37°C. For the zero min time point cells grown at 25°C were labeled with [$^{35}$S]-amino acids for 20 min at 25°C while cells taken after 60 and 120 min of heat treatment were labeled for 15 min at 37°C. Proteins excreted into the culture medium
were separated by SDS-PAGE and radioactive bands were analyzed by using a Phosphorimager. Molecular size markers (in kDa) are indicated at the left of the figure. Each time point shows the proteins secreted into the culture medium for two of the three individual cultures examined as described in “Experimental Procedures.” Bands marked at the right by (o) and (⁎) were not secreted by RH3809 cells.

Fig. 7. **Fur4p associates with lipid rafts.** (A) RH1800 (wild type) or RH3809 (*lcb1-100*) cells were grown overnight in YPD to an A600 of 0.3 at 25°C and then shifted to 37°C for 120 min. Triton X-100 insoluble material was isolated and fractionated by centrifugation on an Optiprep density gradient. Six fractions were collected from the gradient and analyzed by Western blotting using antibodies against Fur4p or against Pma1p, a marker for raft-associated proteins, and Pgk1, a marker for a non-raft protein. (B) Same experiment as in A except that Fur4p was overproduced by transforming RH1800 (wild type) or RH3804 (*lcb1-100*) cells with YEp352F.
Table 1. Strains used in these experiments

| Strain  | Genotype                             | Source       |
|---------|--------------------------------------|--------------|
| RH1800  | MAT a leu2 his4 ura3-52 bar1         | (20)         |
| RH3809  | MATa lcb1-100 leu2 his4 ura3 bar1    | (20)         |
| RH3804  | MATα lcb1-100 leu2 trp1 ura3 lys2 bar1| H. Riezman   |
| NY13    | MATα ura3-52                         | P. Novick    |
| NY17    | MATα ura3 sec6-4                     | P. Novick    |
Table 2. Comparison of sphingolipids in RH1800 and RH3809 cells.

| Strain          | GPI\(^{a,b}\) (cpm/A600) | M(IP)\(_2\)C\(^a\) | IPC + MIPC\(^a\) |
|-----------------|---------------------------|---------------------|-----------------|
| RH1800 (LCB1)   | 234 ± 29                  | 70.5 ± 8            | 46 ± 6          |
| RH3809 (lcb1-100) | 236 ± 13                  | 55 ± 1              | 11 ± 1          |

\(^a\) Values are based on more than 10,000 cpm chromatographed for each sample and represent the average of two experiments ± the standard deviation.

\(^b\) GPI is glycerol-P-inositol, the deaclylation product of phosphatidylinositol.
Fig. 1. Hearn et al.
Fig. 2 Hearn
Fig. 3 Hearn et al.
Fig. 4 Hearn et al.

Minutes at 37°C

CPM/A600/min

0 20 40 60 80 100 120

0 20 40 60 80 100 120

NY13 (Wild Type)

NY17 (sec6-4)

*
Fig. 5 Hearn et al.

Minutes at 37°C

Percent of initial activity

0 20 40 60 80 100

EtOH

PHS

AbA and PHS

*
Fig. 6 Hearn et al.
Fig. 7. Hearn et al.

A

25°C  37°C

RH1800 (WT)

Top  Bottom  Top  Bottom

1  2  3  4  5  6  1  2  3  4  5  6

Fur4p  Pma1p  Pgk1p

RH3809 (lcb1-100)

Fur4p  Pma1p  Pgk1p

B

25°C  37°C

RH1800 (WT)

Top  Bottom  Top  Bottom

1  2  3  4  5  6  1  2  3  4  5  6

Fur4p  Pma1p  Pgk1p

RH3804 (lcb1-100)

Fur4p  Pma1p  Pgk1p
