Heat-induced Elevation of Ceramide in Saccharomyces cerevisiae via de Novo Synthesis*

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Sphingolipid-related metabolites have been implicated as potential signaling molecules in many studies with mammalian cells as well as in some studies with yeast. Our previous work showed that sphingolipid-deficient strains of Saccharomyces cerevisiae are unable to resist a heat shock, indicating that sphingolipids are necessary for surviving heat stress. Recent evidence suggests that one role for the sphingolipid intermediate ceramide may be to act as a second messenger to signal accumulation of the thermoprotectant trehalose. We examine here the mechanism for generating the severalfold increase in ceramide observed during heat shock. As judged by compositional analysis and mass spectrometry, the major ceramides produced during heat shock are similar to those found in complex sphingolipids, a mixture of N-hydroxyhexacosanoyl C18 and C20 phytosphingosines. Since the most studied mechanism for ceramide generation in animal cells is via a phospholipase C-type sphingomyelin hydrolysis, we examined S. cerevisiae for an analogous enzyme. Using [3H]phytosphingosine and [3H]inositol-labeled yeast sphingolipids, a novel membrane-associated phospholipase C-type activity that generated ceramide from inositol-P-ceramide, mannosylinositol-P-ceramide, and mannosylinositol-P,ceramide was demonstrated. The sphingolipid head groups were concomitantly liberated with the expected stoichiometry. However, other data demonstrate that the ceramide generated during heat shock is not likely to be derived by breakdown of complex sphingolipids. For example, the water-soluble fraction of heat-shocked cells showed no increase in any of the sphingolipid head groups, which is inconsistent with complex sphingolipid hydrolysis. Rather, we find that de novo ceramide synthesis involving ceramide synthase appears to be responsible for heat-induced ceramide elevation. In support of this hypothesis, we find that the potent ceramide synthase inhibitor, australifungin, completely inhibits both the heat-induced increase in incorporation of [3H]sphinganine into ceramide as well as the heat-induced increase in ceramide as measured by mass. Thus, heat-induced ceramide most likely arises by temperature activation of the enzymes that generate ceramide precursors, activation of ceramide synthase itself, or both.

Widespread research, primarily in mammalian cells, has focused on sphingolipids as possible mediators of stress responses. Sphingolipid metabolites such as sphingosine, sphingosine-phosphate, and ceramide have been proposed as signaling molecules in a host of cellular processes (for recent reviews, see Refs. 1–4); however, in most cases the precise molecular interactions in a sphingolipid-mediated signaling cascade await definition. Ligand-activated sphingomyelinase activity acting on plasma membrane sphingomyelin is the most studied mechanism for generating the second messenger ceramide (5). Some reports suggest an alternative mechanism; altered ceramide synthase activity produces the ceramide increase seen in apoptosis (6) and arachidonic acid signaling in macrophages (7).

Saccharomyces cerevisiae is an exemplary organism in which to sort out the sphingolipid-related genes and proteins necessary for mounting a response to stress. Not only has the genome sequence been determined, but the sphingolipid composition is relatively simple as compared with mammals. The principal ceramide of S. cerevisiae, phytosphingosine (4-OH sphinganine) N-acylated with an α-OH C18 fatty acid, is phosphodiester-linked to inositol (IPC),1 mannosylinositol (MIPC), or inositolphosphorylmannosylinositol (M(IP)2C) (8). The synthesis of the Saccharomyces cerevisiae sphingolipid hydroxoceramides is schematized in Fig. 1, indicating inhibition of ceramide synthase by the potent antifungal agent australifungin (9).

Sphingolipids were first implicated in stress responses in S. cerevisiae when it was observed that strains unable to make sphingolipids failed to grow under conditions such as high temperature, high osmotic pressure, and low pH, whereas such strains could withstand these stresses when cultured so as to contain sphingolipids (10). Subsequent reports implicated sphingolipids in signaling roles in S. cerevisiae. N-Acetyl sphingosine (C2-ceramide) was shown by some (11, 12) but not all (13) laboratories to inhibit growth (12) via the proposed activation of a protein phosphatase (11, 12).

Shifting the temperature from 24 to 39 °C is known to induce a variety of responses such as heat shock protein synthesis (14) and trehalose accumulation (see Ref. 15 and references cited therein). We recently demonstrated that such a temperature shift caused a severalfold elevation of ceramide (16) as well as a transient increase in sphinganine and phytosphingosine (17). Recent work also indicates that sphingolipids could be involved in the trehalose accumulation response, including the failure to accumulate trehalose in sphingolipid-deficient cells, exogenous sphinganine induction of trehalose accumulation in wild type cells, and exogenous sphinganine induction of the transcription of the TPS2 gene required for trehalose synthesis (17).

In this paper, we examine the biochemical basis for the heat-
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**Preparative Isolation and Analysis of Ceramides from Heat-shocked Cells**

An overnight culture grown at 24 °C (2 liters; $A_{600} = 1.0$) was warmed to 39 °C and incubated with shaking for 40 min, followed by termination with trichloroacetic acid to 5%. The water-washed cell pellet was extracted with 80 ml of chloroform/methanol (1:1) at 50 °C for 30 min and centrifuged while warm, and the supernatant was dried and suspended in 2 ml of chloroform. The extract was applied to a 6-ml silica gel column (Adsorbosil, 100–200 mesh, Applied Science Labs) equilibrated with chloroform, and eluted with 9 ml of chloroform, 9 ml of chloroform/methanol (9:1), and 9 ml of chloroform/methanol (1:1). Ceramide-III assay by HPLC of the three fractions showed 0, 471, and 34 nmol, respectively. Fraction 2 was further purified by HPLC on a 0.45 × 30-cm column of 5-μm Lichrosorb Si60 (Merck) equilibrated with chloroform. The elution schedule (flow rate of 1 ml/min) was 1 min chloroform, 19 min linear gradient between chloroform and chloroform/methanol (75:25). The eluate was monitored with a Varex evaporative light scattering detector, and the fractions expected for ceramide-III were pooled, dried, and dissolved in chloroform. Final purification was on the same Lichrosorb column with isocratic elution with chloroform/methanol (9:1). The final ceramide fraction was analyzed for long chain base and fatty acid (24) as well as assayed by fast atom bombardment mass spectrometry. Part of the sample was benzoylated and separated by HPLC by a scaled up version of the protocol described above for quantitative ceramide analysis. Samples from each of the twin peaks were subjected to electron impact mass spectrometry.

**Mass Spectrometry**

Positive ion fast atom bombardment spectra of underivatized ceramides were measured with a Concept IH (Kratos) two-sector mass spectrometer equipped with an Ion Tech Ltd. sable field gun operating with a xenon gas and set at a resolution of about 1500 at the acceleration voltage of 5.3 kV. Samples (~2 nmol/2 μl) in methylene chloride were added to 3-nitrobenzyl alcohol matrix (2–3 μl) on a 7-mm diameter stainless steel probe tip. Spectra were acquired in a raw data mode, in a 100–210 atomic mass unit range, 3 s per decade, using fragment-free Cs calibration. Spectra representing the best response were averaged and digitally smoothed. Electron impact mass spectra of perbenzoylceramides were measured at 70 eV using a CONCEPT IH (Kratos) two-sector instrument. Samples on the platinum wire were directly introduced to the ion source at 250 °C. Spectra were acquired from 40 to 1400 atomic mass units, 3 s per decade, and the instrument was set to about 2000 atomic mass units resolution for validating.

**Preparation of $^3$H]Phosphosphingolipids**

Sphingolipids labeled with either $[^3]$Hinositol or $[^3]$Hphosphatidylinositol were prepared by metabolic labeling. Ethanol solutions of myo-[2-$^3$H]inositol (1 mCi, NEN Life Science Products) or [4,5-$^3$H]sphinganine (775 × 10$^6$ dpm; prepared as described above) were dried in a sterile culture flask, sonicated with 10 ml of culture medium, inoculated with a starter culture of strain BYH2252 to a starting A$_{600}$ of 0.2 and shaken for 22 h at 30 °C, the A$_{600}$ reaching 11. The cells were treated with trichloroacetic acid (5% final concentration), centrifuged, and washed twice with water. Lipids were extracted by treating each pellet with 2 ml of solvent B (diethylether, 95% ethanol, water, pyridine; 5:15:15:1 (v/v/v/v) containing 0.5 ml/liter concentrated ammonia) for 3 min at 60 °C. Further purification proceeded by slightly different routes.

The solvent B extract from the [4,5-$^3$H]sphinganine-labeled cells was added to a 1-ml column of BioRex 70 resin (H$^+$ form, 200–400 mesh, Bio-Rad) in a Pasteur pipette (packed in water and equilibrated with methanol) and washed with 3 ml of solvent B followed by 2 ml of methanol. The eluates, now free of long chain bases, were combined, dried, and dissolved in 1 ml of solvent B. Acyl ester lipids were deacylated by adding 1 ml of 0.2% KOH in methanol and incubating for 30 min at room temperature. Further work up by adsorption to and elution from a Chelex resin C18 Celite mixture was as described previously (25). The sphingolipid fraction was dried and suspended in 1 ml of chloroform/methanol (1:1) and applied to a 3-ml column of silica gel (Adsorbosil, 100/200 mesh, Applied Sciences, Inc.) equilibrated with chloroform/methanol (1:1) and neutral acetone. The neutral acetone was removed (4,5-$^3$H]sphinganine (~11% of the radioactivity) were eluted with 6 ml of chloroform/methanol (1:1). The [3H]sphingolipids were eluted with 15 ml of solvent A containing three drops of concentrated ammonia/5 ml. Only radioactive sphingolipids were evident by thin layer chromatography (yield, 170 × 10$^6$ dpm).

The solvent B extract from the [3H]inositol-labeled cells was deacylated...
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**Product Analysis of Putative Yeast Membrane Phospholipase C**

**Action on [3H]Phospholipids**

The reaction mixtures (0.3 ml) consisted of 22 mM potassium phosphate, pH 7.0, 2 mM dithiothreitol, 5 mM MgCl₂, 0.6% n-octyl-β-glucopyranoside, 0.22 µg of membrane protein (27 µg). Also added were 10 nmol of unlabeled sphingolipid-IPC (IPC-III, MI, (MIP)²C-III) and equal amounts of radioactivity of the corresponding [3H]inositol-and [3H]sphinganine-labeled sphingolipids. IPC-III, 101,000 cpm; MI, 39,000 cpm; MIP/2C-III, 200,000 cpm. The sphingolipids were dried and suspended by sonic treatment in the assay mixture before the addition of enzyme. After 60 min at 25 °C, the reaction was terminated by 2-min heating (100 °C) followed by adding 30 µl of 0.5 M Na-EDTA, pH 7.1. Aliquots were chromatographed on silica gel paper (solvent C). One-cm zones were subjected to ion exchange chromatography. Water-soluble products were at the origin, ceramides migrated near the solvent front, and sphingolipids migrated at these Rₜ values: IPC-III, 0.65; MI, 0.54; MIP/2C-III, 0.30. Separate µl aliquots of the reaction mixtures were chromatographed on silica gel paper with the solvent chloroform/methanol (19:1.5, v/v) along with ceramide-III standard. Radioactivity was determined for each lane as described in the legend. A. 10 µg of the dry sphinganine was first removed by expressing the reaction mixture, washed twice with water and extracted for sphingolipids with 1 ml of 0.5 M ammonium bicarbonate. Elution was carried out at 1.2 ml/min with 0.3 M ammonium bicarbonate, collecting 20.8 ml fractions, which were analyzed for radioactivity. Peak fraction numbers for labeled products were as follows: inositol, 2; glycerophosphorylinositol, 4; inositol-P and mannosylinositol-P, 6; mannosylinositol-P₂, 1.

**Incorporation of [3H]Sphinganine into Ceramide and Phosphosphingolipids**

About 7.5 membranes units of mid-log phase cells were suspended in 5 ml of fresh medium containing 47 × 10⁶ cpm [3H]sphinganine and incubated at 24 or 39 °C. Aliquots (1.4 µl) were removed and terminated with 0.07 ml of 100% trichloroacetic acid and incubated at least 15 min on ice. The cells were centrifuged and the final supernatants were washed twice with water and extracted for sphingolipids with 1 ml of 0.5 M ammonium bicarbonate. Elution was carried out at 1.2 ml/min with 0.3 M ammonium bicarbonate, collecting 20.8 µl fractions, which were analyzed for radioactivity. Peak fraction numbers for labeled products were as follows: inositol, 2; glycerophosphorylinositol, 4; inositol-P and mannosylinositol-P, 6; mannosylinositol-P₂, 11.

For labeled ceramide analysis, [3H]sphinganine was first removed by applying the solvent B extract to a 0.5 ml column of Bio-Rex 70 (H⁺) resin (200–400 mesh) packed in water and equilibrated with methanol. Elution was carried out with 1.5 ml of solvent A and then with 1 ml of methanol. The combined eluates were dried and dissolved in 0.2 ml of solvent A and subjected to thin layer chromatography (Whatman K5 plates, solvent CHCl₃/methanol, 9:1:1). To each lane, 30 µl of ceramide-III was added. Following radioactivity determination (BioScan apparatus), the plates were sprayed with 10% (w/v) CuSO₄-6H₂O in 8% H₃PO₄ and charred at 160 °C (26) to locate the added ceramide standard. The total radioactivity in the ceramide zone was calculated from the Bio-Rex 70 eluates and from the percentage distribution of radioactivity from the BioScan analysis of the thin layer plates. For phosphosphingolipid analysis, the solvent C extract was dried and deacylated with 0.5 ml of monomethylamine reagent as above followed by thin layer chromatography (200-µ Whatman HP-K plates, solvent C). Each lane contained a mixture of sphingolipid standards, 1–2 µg each of IPC-III, IPC-III, MIP/2C-III, and MIP/2C-III. Following radioactivity determination (BioScan apparatus), the added sphingolipid standards were located by charring (26). The total radioactivity in the ceramide extract was washed with 1 ml of water and dried, and radioactivity was measured. A small no enzyme blank reaction, equivalent to <1% apparent substrate breakdown, was subtracted from the membrane-containing samples to calculate the specific activity.

**Method B**—The reaction was started by the addition of 3 ml of chloroform/methanol (1:1) to the centrifuged supernatant was added to a 1-ml column of AG4-X4 (acetate form, 100–200 mesh, Bio-Rad; packed in water and equilibrated with methanol) and eluted with 3 ml of chloroform/methanol (1:1). The substrates bound to the resin. The eluted ceramide was dried and assayed for radioactivity. A no enzyme blank value was subtracted.

**Turnover of Inositol-labeled Lipids**

An overnight log phase culture was transferred to 15 ml of fresh medium containing 1.5 mM of myo-[2-3H]inositol (American Radiolabeled Chemicals, Inc.) to give starting A₀₋₀ = 0.2 and cultured for 6 h at 24 °C. The cells were rapidly resuspended in 15 ml of fresh nonradioactive medium and divided in three parts. One part (zero time) was centrifuged, and the pellet was washed with 1 ml of 0.1 M NaClO₄. The other two parts were incubated at 24 and 39 °C for 20 min followed by rapid centrifugation and quenching of the cell pellets with HClO₄ as described (26). Extracts and culture medium fractions, which were filtered (0.2-µm Teflon Acrodiscs, CR, Gelman Sciences). After standing for 15 min at 0 °C, the HClO₄-treated cell pellets were frozen and thawed twice in dry ice/ethanol and centrifuged at 0 °C. The supernatants were slowly neutralized (chlorophenol red pH indicator) at 0 °C with 2.6 mM KOH and centrifuged, and the final supernatants were washed twice with water and extracted for sphingolipids with 1 ml of solvent B for 30 min at 60 °C, followed by centrifugation while warm. The extract was dried and deacylated by treatment with 0.5 ml of monomethylamine reagent (28) for 30 min at 50 °C. After evaporation of the reagent, the sample was dissolved in 1 ml of solvent A, and aliquots were chromatographed on silica gel paper (solvent C). One-cm zones from each lane were subjected to ion exchange chromatography. Radioactivity was determined for each lane as described in the legend. A. 10 µg of the dry sphinganine was first removed by expressing the reaction mixture, washed twice with water and extracted for sphingolipids with 1 ml of 0.5 M ammonium bicarbonate. Elution was carried out at 1.2 ml/min with 0.3 M ammonium bicarbonate, collecting 20.8 µl fractions, which were analyzed for radioactivity. Peak fraction numbers for labeled products were as follows: inositol, 2; glycerophosphorylinositol, 4; inositol-P and mannosylaminoinositol-P, 6; mannosylinositol-P₂, 11.

For labeled ceramide analysis, [3H]sphinganine was first removed by applying the solvent B extract to a 0.5 ml column of Bio-Rex 70 (H⁺) resin (200–400 mesh) packed in water and equilibrated with methanol. Elution was carried out with 1.5 ml of solvent A and then with 1 ml of methanol. The combined eluates were dried and dissolved in 0.2 ml of solvent A and subjected to thin layer chromatography (Whatman K5 plates, solvent CHCl₃/methanol, 9:1:1). To each lane, 30 µl of ceramide-III was added. Following radioactivity determination (BioScan apparatus), the plates were sprayed with 10% (w/v) CuSO₄-6H₂O in 8% H₃PO₄ and charred at 160 °C (26) to locate the added ceramide standard. The total radioactivity in the ceramide zone was calculated from the Bio-Rex 70 eluates and from the percentage distribution of radioactivity from the BioScan analysis of the thin layer plates. For phospholipid analysis, the solvent C extract was dried and deacylated with 0.5 ml of monomethylamine reagent as above followed by thin layer chromatography (200-µ Whatman HP-K plates, solvent C). Each lane contained a mixture of sphingolipid standards, 1–2 µg each of IPC-III, IPC-III, MIP/2C-III, and MIP/2C-III. Following radioactivity determination (BioScan apparatus), the added sphingolipid standards were located by charring (26). The total radioactivity in the ceramide extract was washed with 1 ml of water and dried, and radioactivity was measured. A small no enzyme blank reaction, equivalent to <1% apparent substrate breakdown, was subtracted from the membrane-containing samples to calculate the specific activity.

**Method B**—The reaction was started by the addition of 3 ml of chloroform/methanol (1:1) to the centrifuged supernatant. The supernatant was added to a 1-ml column of AG4-X4 (acetate form, 100–200 mesh, Bio-Rad; packed in water and equilibrated with methanol) and eluted with 3 ml of chloroform/methanol (1:1). The substrates bound to the resin. The eluted ceramide was dried and assayed for radioactivity. A no enzyme blank value was subtracted.
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Effects of Australifungin and Cycloheximide on Conversion of [3H]Sphinganine to Ceramide and Phosphosphingolipids

A log phase culture grown at 24 °C was transferred to fresh medium without Tergitol (A_{660} = 1.7), and 4.5-ml samples were incubated for 10 min at 24 °C after the addition of 3 μl of australifungin (500 μg/ml ethanol) or 3 μl of ethanol to the controls. After the addition of 0.5 ml of 0.5% Tergitol containing 2.5 × 10^6 dpm [3H]sphinganine, incubation was continued at 24 and 39 °C. At 20, 40, and 60 min, 1.4-ml aliquots were quenched with 0.07 ml of 100% trichloroacetic acid. Another experiment was carried out as above, except cycloheximide, 1 mM final concentration, was added at zero time and after 30 min of incubation at 24 or 39 °C. Lipid extracts were benzoylated and subjected to HPLC as described above.

RESULTS

Evidence for Heat Induction of Two Molecular Species of Ceramide—S. cerevisiae cells were cultured at 24 °C and switched to 39 °C, and their extracted lipids were derivatized and separated by HPLC. It can be seen (Fig. 2) that a significant increase occurs at 39 but not at 24 °C in a double peak that is sustained for at least 2 h (Fig. 3). The 4–5-fold heat-induced ceramide elevation appears to be specific to heat stress, since neither high osmotic pressure (Fig. 3B) nor low pH (Fig. 3B) at 24 °C resulted in marked elevation of ceramide.

Enzymatic Hydrolysis of Yeast Phosphosphingolipids Can Generate Ceramide—Activation of a sphingomyelinase activity toward yeast phosphosphingolipids has not been previously reported. We sought evidence for the existence of a comparable phosphodiesterase, with phospholipase C specificity, that would hydrolyze yeast phosphoinositol sphingolipids to generate ceramide. Ceramide (N-hydroxyhexacosanoylphosphosphingosine)-labeled sphingolipids were isolated from cells metabolically labeled with [3H]sphinganine and used as substrates. In one enzyme assay (method A), the radiolabeled ceramide product was extracted with methyl tert-butylerther. In an alternate assay (method B), the solvent-treated assay mixture was chromatographed on a small anion exchange column, which retained the acidic substrates but not the free ceramides.

[3H]Ceramide was released from [3H]IPC, [3H]MIPC, and [3H]M(IP)2C by incubation with crude membrane preparations in a reaction that absolutely required octyl glucoside and MgCl2 (not shown). Ceramide release was reasonably linear with time, and protein concentration and optimum hydrolysis activity was obtained with 2–3 mol % of M(IP)2C in the mixed micelles, bulk concentration 33 μM (Fig. 4). The pH optimum for the reaction was about 6–6.5 (Fig. 4).

Product Analysis of Yeast Membrane Phosphodiesterase Action on [3H]Phosphosphingolipids—Since phosphodiesterase activity toward yeast phosphosphingolipids has not been previously reported, it seemed essential to define the stoichiometry and nature of the products in order to establish whether hydrolysis is between the phosphorus and ceramide (phospholipase C) or between the phosphorus and the inositol (phospholipase D), the latter generating phosphoceramide that would have to undergo further hydrolysis by a phosphatase to yield ceramide. All of the evidence described below is consistent with membranes containing an enzyme(s) with phospholipase C-

### Table I

| Isolated         | Perbenzoylated | Peak I | Peak II |
|------------------|----------------|--------|---------|
| Putative composition |                |        |         |
| Fatty acid       | OH26:0         | C-18   | C-20    |
| Phytosphingosine | OH26:0         | C-18   | C-20    |
| Chemical composition found | OH-fatty acids/phytosphingosines | 0.96    |         |
| Mass spectrometry | (major molecular ions) | MH⁺ expected (FAB) 712.7 740.7 | MH⁺ found    |
|                  |                 | 712.4 740.5 | 1155.8 1127.8 |

Stress—We hypothesized that mutant strains lacking sphingolipids cannot grow at low pH, in high salt, or at high temperature (10) because they are unable to generate sphingolipid second messengers such as ceramide. To test this hypothesis, we examined wild type cells for changes in ceramide following stress. A rise in ceramide concentration can be detected after 10 min of heat treatment at 39 °C. The rise peaks after 30–40 min and is sustained for at least 2 h (Fig. 3). The 4–5-fold heat-induced ceramide elevation appears to be specific to heat stress, since neither high osmotic pressure (Fig. 3A) nor low pH (Fig. 3B) at 24 °C resulted in marked elevation of ceramide.

**FIG. 2. Assay of ceramide (Cer-III Std) in heat-shocked cells.**

Aliquots (50 ml) of a culture grown at 24 °C to an A_{660} of 0.57 were removed at zero time and after 30 min of incubation at 24 or 39 °C. Lysates were benzoylated and subjected to HPLC as described under “Experimental Procedures.”

The phosphosphingolipid zones was calculated from the deacylated solvents extracts and from the percentage distribution of radioactivity (Bioscan apparatus) on the thin layer plates.
type phosphodiesterase activities catalyzing the following reactions.

\[ \text{M(IP)}_2\text{C} \rightarrow \text{Ceramide} + \text{inositol-P-mannose-inositol-P} \]

\[ \text{M(IP)}_2\text{C} \rightarrow \text{MIPC} + \text{inositol-P} \]

\[ \text{MIPC} \rightarrow \text{Ceramide} + \text{mannose-inositol-P} \]

\[ \text{IPC} \rightarrow \text{Ceramide} + \text{inositol-P} \]

**REACTIONS 1–4**

We first reacted sphingolipid substrates containing equal radioactivity in their ceramide and inositol portion and chromatographed the entire reaction mixture on silica gel-impregnated paper. In this system, the water soluble product(s) remain at the origin, while ceramide and other sphingolipids migrate at the RFs indicated under “Experimental Procedures.” In the case of IPC-III, equal amounts of radioactivity were found in the ceramide and origin regions, consistent with phosphodiesterase activity (Table II). In the case of M(IP)₂C, in addition to ceramide, some MIPC was formed, requiring that some inositol-P be one of the polar products. The ratio of ceramide to total polar product radioactivity was as expected for the action of a phosphodiesterase(s) cleaving M(IP)₂C to yield free ceramide as well as yielding equimolar amounts of inositol-P and MIPC (Table II). The observed stoichiometry is consistent with a phosphodiesterase acting on the phosphosphingolipids.

Evidence for a phospholipase C-type mechanism for sphingolipid hydrolysis was obtained by showing that the polar products had alkaline phosphatase-susceptible phosphomannoester groups. The polar products generated from [³H]inositol and [³H]phosphatidylcholine were reacted with yeast membranes for 60 min, the reaction mixture was subjected to chromatography on silica gel-impregnated paper, and the distribution of radioactivity in each lane was measured as described under “Experimental Procedures.”

**FIG. 4.** Ceramide formation by reaction of [³H]M(IP)₂C and [³H]IPC with putative membrane phospholipase C. [³H]M(IP)₂C was reacted with yeast membranes and processed as per “Experimental Procedures.” A and B, method B; C, method A; D, [³H]IPC-III was the substrate and was processed as described in the legend to Table II.

**TABLE II**

| Substrates     | Distribution of radioactivity | Ceramide/polar product(s) ratio |
|----------------|------------------------------|-------------------------------|
|                | %                            |                               |
| IPC-III        | 6.5                          | 6.5                           | 87.0 | 1.00 | 1.00 |
| M(IP)₂C-III    | 10.8                         | 2.3                           | 11.7 | 75.3 | 0.93 | 0.92 |

*The expected polar product percentage was equal to the ceramide plus one-third of the MIPC.*
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Analysis of the water-soluble products formed by the action of yeast membranes on IPC and M(IP)₂C

Table III

| Substrates | Enzyme product, approximate net charge | Distribution of tritium after anion exchange chromatography |
|------------|--------------------------------------|----------------------------------------------------------|
|            | 0                                    | %                                                        |
| IPC-III    | -1                                   | 85.9                                                     |
| M(IP)₂C-III| 0                                    | 0                                                        |

Ammonolysis of the water-soluble products produced by the action of putative phospholipase C on [³H]M(IP)₂C

Table IV

| Compound ammonolysed | Mannosylinositol/inositol ratio |
|----------------------|--------------------------------|
| M(IP)₂C-III (starting lipid) | 0.86 ± 0.08 |
| M(IP)₂C-III, charge = -2 peak | 0.89 ± 0.03 |
| M(IP)₂C-III, charge = -3 peak | 0.78 ± 0.05 |

Migrated to the net charge -1 region (Table III), consistent with the reaction inositol-P-mannose-inositol-P → inositol-P-mannose-inositol + P₁. The identities of the two radioactive major M(IP)₂C products (Table III) were further established by subjecting each to ammonolysis conditions that hydrolyze all phosphate bonds, leaving the mannose-inositol glycosidic bond intact (29). The radioactive ammonolysis products were resolved by TLC with the radioactivity being accounted for as inositol and mannosylinositol. Table IV shows that each major peak gave the same ratio of mannosylinositol to inositol counts as the original M(IP)₂C. These data are consistent with the interpretation that the charge −3 peak (Table III) composition was inositol-P-mannose-inositol-P and that the charge −2 peak consisted of about equal amounts of inositol-P and mannose-inositol-P. The digestion of M(IP)₂C (Table III) was carried out for much longer than the experiment described in Table IV (see “Experimental Procedures”), probably accounting for a higher proportion of the reaction M(IP)₂C → MIPC + inositol-P.

Table V

| Does heat-induced ceramide elevation arise from catabolism of inositol sphingolipids? |
|--------------------------------------|--------------------------------------|
| Cells                               | Change in tritium between zero time and 20 min |
| HClO₄-soluble                       | Tritium at zero time |
| Inositol                            | 24 °C | 39 °C |
| Acridic sphingolipid catabolitesa    | 180   | 340   |
| Inositol lipids                     | 1577  | 232   |
| IP₃/MIPC                            | 2500  | 457   |
| Phosphatidylinositol                | 6128  | 1502  |
| Culture medium                      |       |       |
| Glycerophosphorylinositol           | 0     | 546   |
| Acridic sphingolipid catabolitesb   | 0     | 200   |
| Increase expectedc in acidic catabolite(s) assuming ceramide elevation at 39 °C is due to sphingolipid breakdown | 258   |       |

a Sum of radioactivity in the eluate regions expected for inositol-P, mannosyl-inositol-P, and inositol-P-mannose-inositol-P.

b Ceramide increase at 39 °C is due to sphingolipid breakdown.

Look for peaks of radioactivity that increase in the 39 versus 24 °C samples at elution volumes expected for the sphingolipid head groups (Table III). Based on the radioactivity in the total sphingolipid head groups (Table V) at zero time (4,077,000 cpm) and the increase in ceramide expected to result from heat treatment (Fig. 3) equivalent to breakdown of about 6% of the sphingolipid (see legend to Table V), we can calculate that the acidic sphingolipid catabolites should increase by about 258,000 cpm.

Further support for this conclusion was obtained with a mutant (RCD113) defective in M(IP)₂C synthesis (22), which...
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Heat-induced ceramide elevation in a strain devoid of M(IP)_2C

Cells cultured at 24 °C to an A650 of 0.8 were incubated for 20 min at 24 and 39 °C. Ceramide-III was measured by HPLC after perbenzoylation as described under "Experimental Procedures."

| Strains       | Ceramide-III content | Zero time 24 °C, 20 min | 39 °C, 20 min |
|---------------|----------------------|-------------------------|--------------|
| YPH250 (wild type) | 25.6                 | 26.7                    | 89.4         |
| RCD113 (Δipt1)  | 38.8                 | 32.5                    | 94.2         |

Fig. 5. Effect of exogenous phytosphingosine (PHS) on ceramide levels. Cultures (100 ml) grown at 24 °C to an A650 of 0.8 were mixed with 10 ml of fresh medium with or without phytosphingosine to yield 0, 20, and 100 μM. Incubation was continued at 24 °C (open circles) or 39 °C (closed circles) with samples processed at 0, 15, and 30 min for ceramide-III analysis (perbenzoyl derivatives, "Experimental Procedures").

makes no detectable M(IP)_2C due to the deletion of the IPT1 gene but accumulates increased levels of MIPC. Strain RCD113 gave a heat-induced ceramide response equivalent to its cognate wild type strain (Table VI). Thus, M(IP)_2C breakdown cannot be the source of the elevated ceramide level observed during heat shock.

Is de Novo Synthesis of Ceramide Responsible for the Heat-induced Increase?—In view of all of the results, we considered whether increased de novo synthesis accounts for heat-induced increases in ceramide. We showed previously (17) that a change from 24 to 39 °C results in a temporary elevation of the concentration of sphinganine and phytosphingosine. We therefore determined if exogenous long chain base alone could increase ceramide levels. When phytosphingosine was added to cultures at 24 °C, ceramide did not increase to the level achieved by addition of 1 μM sphinganine at 39 °C (Fig. 5). A similar experiment carried out with 50 μM L-sphinganine at 24 °C induced little increase in ceramide above the untreated control (data not shown). We conclude that increased long chain base synthesis alone is insufficient to account for the ceramide increase observed at 39 °C.

Further evidence that de novo ceramide synthesis is responsible for heat-induced ceramide accumulation was sought by studying the incorporation of [3H]sphinganine into ceramide at 24 and 39 °C in the presence of 0.3 μg/ml australifungin added at zero time (dotted lines) and without australifungin (solid lines). B, accumulation of radiolabeled ceramide-III in the presence of 1 μM cycloheximide (dotted lines) or in the absence of cycloheximide (solid lines).

Table VI

| Incubation time, temperature | Ceramide-III pmol/A650 unit |
|-----------------------------|----------------------------|
| Zero time 24 °C, 20 min     | 22.7 ± 4.7                 |
| 30 min, 24 °C               | 20.2 ± 0.8                 |
| 30 min, 39 °C               | 106 ± 1.9                  |
| 30 min, 39 °C + australifungin | 5.6 ± 0.05                |

If the 39 °C induced increase in ceramide were due to increased synthesis of the enzyme ceramide synthase, then the enhanced conversion of [3H]sphinganine to ceramide should be blocked by cycloheximide. Cycloheximide (1 μM) did not inhibit ceramide synthesis at either temperature (Fig. 6B) but actually increased the extent of labeling; thus, it is unlikely that increased synthesis of ceramide synthase or any other protein mediates the temperature-induced synthesis of ceramide.

Another explanation for enhanced ceramide accumulation is a reduction in the rate of conversion of ceramide to complex sphingolipids following a temperature shift, for example, by inhibition of IPC synthase (Fig. 1). However, the temperature shift from 24 to 39 °C does not inhibit but rather increases total radiolabeling of complex sphingolipids by [3H]sphinganine, although not to the extent that it enhances ceramide labeling; as expected, sphingolipid labeling is abolished by australifungin (Fig. 6C).

Finally, although australifungin is a potent ceramide syn-
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De Novo Synthesis Is Responsible for Heat-induced Elevation of Ceramide—Our experiments demonstrate that de novo synthesis, not breakdown of sphingolipids, is the primary mechanism for generating an increased level of ceramide following a shift from 24 to 39 °C. To show that de novo synthesis of ceramide via ceramide synthase (Fig. 1) was essential for an increased ceramide, we employed australafugin, a potent antifungal drug shown to be an inhibitor of ceramide synthase (9). The heat-induced increase in ceramide as measured directly by mass as well as by incorporation of [3H]sphinganine into ceramide, were completely inhibited by australafugin (Table VII, Fig. 6), thus identifying the essentiality of ceramide synthase for the heat-induced ceramide accumulation.

The question of what reaction(s) leading to increased ceramide are affected by temperature elevation is complex and largely unanswered. One possible explanation could be differential temperature effects on the rate constants of the enzymatic reactions leading to ceramide synthesis and to its further metabolism. An additional explanation is that elevated temperature could lead to alterations of the amount and/or structure of one or more enzymes of ceramide metabolism. Increased enzyme synthesis does not play a role as judged by the lack of effect of cycloheximide on ceramide synthesis (Fig. 6B). A heat-induced decreased rate of ceramide conversion to IPC and more complex sphingolipids, which could explain ceramide accumulation, is also not evident from the experiment measuring the incorporation of [3H]sphinganine into sphingolipids (Fig. 6C).

Thus, the enzymes that are likely candidates for temperature regulation are those involved in generating ceramide precursors as well as ceramide synthase itself. Earlier work showed (17) that heat shock causes a rapid and temporary rise in the concentration of sphinganine and phytosphingosine, precursors of ceramide. However, increased exogenous long chain base by itself appears to be inadequate to account for increased ceramide, since exogenous long chain bases added in excess at 24 °C did not stimulate ceramide concentrations to the level achieved at 39 °C (Fig. 5). The mechanism of heat-induced ceramide accumulation merits further analysis, especially since exogenous sphinganine activates trehalose accumulation at 24 °C via gene activation (17), thus implicating yeast sphingolipids in a well known stress response.

Ligand activation of a sphingomyelinase, the “sphingomyelin cycle” (5), has been the most studied reaction to account for stress-induced ceramide generation in animal cells. However, two studies with animal cells implicated ceramide synthase in ceramide generation. Daunorubicin-induced apoptosis and ceramide elevation were prevented by the ceramide synthase inhibitor, fumonisin B1 (6); however, other workers claim sphingomyelin hydrolysis is associated with daunorubicin-induced apoptosis (30). Fumonisin has been reported to inhibit ceramide elevation associated with macrophage activation (7).

Two studies in animals cells have observed heat shock-induced elevation in ceramide by as yet undefined mechanisms (31, 32). Future work needs to be directed at the unknown mechanism(s) of temperature regulation of ceramide synthase activity in both animal cells and yeast.

Phospholipase C Type Activity in Yeast Utilizing Phosphoinositol-containing Sphingolipids—Generation of ceramides by activation of a sphingomyelinase,

Choline-P-ceramide + H2O → Choline-P + ceramide

Reaction 5

is the predominant paradigm in mammalian cells for the formation of mediators in various signaling pathways with diverse outcomes (1–4). We therefore looked for a comparable phosphodiesterase activity in yeast to explain the heat-induced increase in ceramide. Our data suggest the existence of one or more phosphodiesterases in S. cerevisiae membranes capable of catalyzing the hydrolysis of yeast sphingolipids to yield ceramide,

R-Inositol-P-ceramide + H2O → R-inositol-P + ceramide

Reaction 6

where R represents hydrogen, mannose, or inositol-P-(mannose). With sphingolipids containing [3H]ceramide as well as [3H]inositol in the head groups, the above stoichiometry was demonstrated (Tables II–IV). Furthermore, the ceramides from heat-shocked cells were isolated, and their chemical composition was confirmed by chemical analysis and by mass spectrometry (Table I). These analyses as well as ceramide analysis in the various heat shock experiments were all performed by chemical methods, a noteworthy observation in light of the recent challenge of the validity of ceramide measurements made by many investigators employing the enzyme diacylglycerol kinase for ceramide analysis (18).

Another phosphodiesterase with activity toward substrates containing ceramide has been described in S. cerevisiae. Ella et al. (13) characterized a sphingomyelinase activity, partially purified from S. cerevisiae membranes, capable of generating ceramide from sphingomyelin. However, this enzyme preparation had no activity toward yeast phosphoinositol sphingolipids. This enzyme was dependent on a divalent cation, as was the sphingolipase activity we describe, but was inhibited by octyl glucoside and other detergents unlike our enzyme activity. It should be noted that sphingomyelin has not yet been reported to occur in S. cerevisiae. The sphingomyelinase of Ella et al. (13) is not likely to be a phosphoinositol sphingolipid hydrolase.

Several groups (33–36) have reported on a putative phospholipase C gene (PLC1) in S. cerevisiae, which upon deletion, results reportedly in differing phenotypes such as lethality or very slow, temperature-sensitive growth, etc. PLC1 was purified as a soluble enzyme after overexpression and was shown to catalyze the following reactions (36).

Phosphatidylinositol + H2O → Inositol-P + diacylglycerol

Phosphatidylinositol 4,5-bis-P + H2O → Inositol 1,4,5-tris-P + diacylglycerol

Reactions 7 and 8

We tested a sample of this enzyme (generously supplied by Dr. Jeremy Thorner) and found it to be without effect on yeast phosphoinositol sphingolipids when assayed (data not shown) as described (36). Furthermore, we assayed hydrolysis of [3H]IPC and [3H]M(IP)2C with membranes prepared from strain YJF132 (36) carrying a plc1 deletion as well as from the cognate wild type strain YJF131 (36). The plc1-deleted strain had about 50% of the sphingolipid hydrolase wild type activities. Whether the lowered activity is related to the very slow growth of the mutant strain and/or some indirect effect of the...
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lack of plc1p on the regulation of sphingolipid phospholipase activity is unclear. Nonetheless, it is clear that substantial sphingolipid hydrolase activity remains in the plc1 deletion strain, and thus plc1 is unlikely to code for sphingolipid hydrolase activity.

We previously examined the turnover of [3H]inositol labeled lipids with uniformly labeled S. cerevisiae cells transferred to nonradioactive growth medium. Although a large decrease in the phosphatidylinositol pool could be readily observed, consonant with its conversion to sphingolipids and extracellular glycerophosphoinositol, no decrease in the total sphingolipid content with its conversion to sphingolipids and extracellular lipid head groups masking any accumulation. In conclusion, the absence of sphingolipid head group accumulation and the observed australifungin-sensitive nature of ceramide elevation make de novo ceramide synthesis the most likely mechanism for heat-induced ceramide elevation.

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Heat-induced Elevation of Ceramide in *Saccharomyces cerevisiae* via de Novo Synthesis

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