The Isolation and Characterization of a Mutant Strain of *Saccharomyces cerevisiae* That Requires a Long Chain Base for Growth and for Synthesis of Phosphosphingolipids*

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A mutant of *Saccharomyces cerevisiae* has been obtained that shows an absolute growth requirement for long chain bases found in sphingolipids. In the absence of a long chain base, the cells are unable to synthesize the phosphinositol-containing sphingolipids characteristic of yeast. These results suggest that one or more of the yeast sphingolipids plays a vital biological role.

Sphingolipids are widespread membrane constituents in animals (1), higher plants (2), and fungi (3); they are rare but not unknown in bacteria (4). The apolar portion of all these lipids is an N-fatty acyl long chain base (ceramide) to which is attached a wide variety of polar head groups, the exact nature of which depends on the class of the organism. Although their ubiquity implies a vital biological role, no specific molecular functions have been ascribed to any sphingolipid; in animals, interest actively centers on cell-cell interaction and recognition phenomena in growth and differentiation (5). *Saccharomyces cerevisiae* contain a unique set of sphingolipids related to those of other fungi and plants (6) but not reported in animals. Schematically, these are IPC, MIPC, and M(IP)C.

We report herein the discovery of a mutant of *S. cerevisiae* that has an absolute requirement for a long chain base for sphingolipid synthesis and for growth; this suggests a vital role for one or more of the yeast sphingolipids.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**—An inositol auxotroph of *S. cerevisiae* MC6A was obtained from Dr. Susan Henry (Albert Einstein College of Medicine) and is referred to as wild type. Cells were cultured at 30 °C with shaking, and turbidity was monitored by absorbance measurement at 650 nm (1 cm) with a Zeiss PMQ-2 spectrophotometer. Basal medium consisted of Difco Vitamin-Free Yeast Nitrogen Base (16.7 g/l), glycylglycine (pH 3.2) (0.05 M), and for Synthesis of Phosphosphingolipids*.

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*The abbreviations used are: IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; M(IP)C, mannosylinositol phosphorylceramide; III and IV refer to the presence of monohydroxy (III) and dihydroxy (IV) fatty acids in the ceramide portion of these lipids.

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**RESULTS**

**Isolation of the Mutant**—The possibility that an auxotrophic mutant could be obtained stemmed from our observation that wild type *S. cerevisiae* cells growing on the agar solidified culture medium described under “Materials and Methods” could take up labeled dihydrophosphogine and incorporate it with high efficiency into all the yeast sphingolipids. Selection of a long chain base auxotroph by the inositol-less death procedure (7) was unsuccessful. Therefore, we screened over 10⁶ mutagenized colonies for long chain base auxotrophs and found several with growth comparable to wild type in the presence of long chain base. One of these, Lcb-20A, is the subject of this communication.

**Growth Requirement for Long Chain Base**—In the presence of long chain base, strain Lcb-20A grows at the same rate as the parental strain (Fig. 1). In the absence of long chain base, this mutant undergoes a doubling of its turbidity at the same rate as the parental strain; after another doubling at a slower rate, no further increase in turbidity is observed. The growth yield obtained with limiting amounts of several long chain

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bases was examined (Fig. 2). Phytosphingosine, the natural product, was significantly more effective than either the erythro- or threo-isomers of DL-dihydrosphingosine. If only one of the DL-pair is effective, then the threo- and erythro-dihydrosphingosines would be about as effective as phytosphingosine. D-Sphingosine supported only marginal growth.

**Synthesis of Phospholipids by Lcb-20A**—We wished to verify that long chain base auxotrophy was associated with an inability to make the complex phosphosphingolipids of *S. cerevisiae* rather than a correction of some other unrelated defect. Phospholipid synthesis was studied by measuring the incorporation of $^{32}$P into the lipids of the mutant and parental strain cultured in the presence and absence of dihydrosphingosine. The data in Table I indicate that in the presence of DL-erythro-dihydrosphingosine, incorporation of $^{32}$P into both total acyl ester phospholipid and alkali-stable phospholipids proceeds at the same rates in parental and mutant strains throughout the course of exponential growth. Acyl ester phospholipid synthesis with the mutant is scarcely diminished during the initial 5.0 h of long chain base starvation but declines sharply thereafter. In contrast, total alkali-stable phospholipid synthesis is sharply depressed between 0.5 and 2.5 h post-starvation and declines to less than 10% of the long chain base fed control between 5.5 and 7.5 h post-starvation (Fig. 1) even though the turbidity is still 90% of the control after 7.5 h (Fig. 1). Curiously, the absence of long chain base appears to significantly stimulate phospholipid synthesis in the parental strain.

Incorporation of $^{32}$P into individual phospholipids was also measured during culture of the mutant in the presence and absence of DL-erythro-dihydrosphingosine. The acyl ester phospholipids were estimated after two-dimensional chromatography on silica gel-impregnated paper. From 0.5 to 2.5 h, no significant effect of long chain base starvation could be detected in the synthesis of the six major acyl ester phospholipids (Table II). After 5 h of starvation, significant depression in the rate of synthesis of all acyl ester phospholipids was observed ranging from a 73% decrease for phosphatidylcho
The synthesis of phospholipids in mutant cells cultured in the presence and absence of DL-erythro-dihydrosphingosine

Lcb-20A cells were cultured in the presence and absence of DL-erythro-dihydrosphingosine and pulsed with \(^{32}\)P, as described in the legend to Table I. The lipid extract freed of nonlipid impurities was chromatographed on silica gel-impregnated paper as described under “Materials and Methods.” The rate of incorporation was calculated from the specific activity of \(^{32}\)P in the culture medium and the absorbance of the culture at the end of the pulse period.

| Pulse time | +DL-Erythro-dihydrosphingosine | -DL-Erythro-dihydrosphingosine | +DL-Erythro-dihydrosphingosine |
|------------|---------------------------------|-------------------------------|--------------------------------|
| 0.5-2.5 h  | Phosphatidylserine               | Phosphatidylglycerol          | Phosphatidylethanolamine       |
|            | Phosphatidylinositol             | Cardiolipin                   | Cardiolipin                   |
| 5.5-7.5 h  | Phosphatidylglycerol             | Phosphatidylethanolamine      | Phosphatidylglycerol           |

\(^{32}\)P \rightarrow \text{lipid} (pmol/h/absorbance/ml culture)

| Phosphatidylserine | Phosphatidylglycerol | Phosphatidylethanolamine |
|-------------------|----------------------|-------------------------|
| +DL               | -DL                  | +DL                     |
| Phosphatidylserine| 660                  | 669                     | 675                       |
| Phosphatidylglycerol| 239                 | 273                     | 240                       |
| Phosphatidylethanolamine| 145            | 166                     | 138                       |

DISCUSSION

Based on in vivo and in vitro experiments (9, 11, 12), the pathway of sphingolipid synthesis in yeast was postulated to be a ceramide → IPC → MIPC → M(IP)_2C, with phosphatidylinositol and GDP mannosase as the phosphoinositol and mannosase donors, respectively. With this pathway in mind, one can expect that starvation for long chain base would first affect the incorporation of \(^{32}\)P into IPC and MIPC as ceramide is depleted and that incorporation into M(IP)_2C would be affected last because \(^{32}\)P phosphatidylinositol could react with pre-existing unlabeled MIPC to give labeled M(IP)_2C. This conforms with the data observed in Fig. 3 and Table II. Since the sphingolipid products formed by culture of the mutant with dihydrosphingosine all appear to contain phytosphingosine (Fig. 3), the mutant must actively convert dihydrosphingosine to phytosphingosine (as does the parental strain). It is logical to speculate that the mutant is blocked in the formation of long chain base with the synthetic defect either in the formation of ketodihydrosphingosine or in its reduction to dihydrosphingosine. The observation that the unnatural three-isomer of dihydrosphingosine supports growth (Fig. 2) might be interpreted to mean that the cells can somehow epimerize the three- and the erythro-isomers; if this is the case, then this may rule out reduction of ketodihydrosphingosine as the affected step. Direct enzyme assays should resolve these questions. The ineffectiveness of D-sphingosine, the animal long chain base, in supporting

line to a 54% decrease for phosphatidylethanolamine. The synthesis of individual alkali-stable phosphosphingolipids was evaluated after resolution by column chromatography (Fig. 3). The major sphingolipid species resolved contain phytosphingosine (\(\text{D-ribo-1,3,4-trihydroxy-2-amino-octadecane}\)) and a monohydroxy long chain fatty acid (IPC-III, M(IP)_2C-III) or a dihydroxy long chain fatty acid (IPC-IV, MIPC-IV, M(IP)_2C-IV). From 0.5 to 2.5 h post-starvation, incorporation of \(^{32}\)P into MIPC species is less than 5% of the long chain base fed control (Fig. 3 and Table II), and strong inhibition of incorporation is observed for the IPCs (80%) and the M(IP)_2Cs (67%). During the 5.5-7.5 h post-starvation interval, incorporation into both IPCs and MIPCs is less than 2% of fed control and incorporation into M(IP)_2Cs about 10% of the fed control.

Thus, with Lcb-20A, an early, severe, and differential inhibition of phosphosphingolipid synthesis is observed as a consequence of starvation for long chain base. The inhibition of sphingolipid synthesis precedes the restriction of growth.

\(^{2}\)G. B. Wells and R. L. Lester, unpublished observations.
growth (Fig. 2) possibly suggests a tight specificity in its transport or in its conversion to complex sphingolipids; alternatively, yeast sphingolipids constructed with sphingosine may function poorly.

To our knowledge, this is the first mutant of this kind studied, and this study strongly suggests that sphingolipids are vital substances. We hope that a study of the molecular pathology of long chain base starvation in such mutants may shed light on the specific biological function(s) of the sphingolipids.

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