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Characterization of sphingosine kinase (SK) activity in *Saccharomyces cerevisiae* and isolation of SK-deficient mutants

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Sphingosine kinase (SK) catalyses the phosphorylation of sphingosine to generate sphingosine 1-phosphate, which is a second messenger involved in the proliferative responses of mammalian cells. Although the yeast *Saccharomyces cerevisiae* has similar phosphorylated sphingoid bases which appear to be involved in growth regulation and the response to stress, SK activity had not been previously demonstrated in yeast. In this study, an *in vitro* system was set up to characterize yeast SK activity. Activity was detected in the cytosol at neutral pH and 37 °C. Yeast SK phosphorylated the sphingoid bases sphingosine, dihydrosphingosine and phytosphingosine. (D,L)-*threo*-dihydrosphingosine, an inhibitor of mammalian SK, did not inhibit the yeast enzyme. Unique properties of yeast SK were an optimal temperature of 43 °C, and *in vivo* activation during nutrient deprivation. Spontaneous mutants with diminished SK activity were isolated utilizing a screen for resistance to sphingosine in a sphingosine-phosphate-lyase deletion background. Abnormal growth and heat sensitivity were observed in these mutants. These findings suggest that SK may function as a stress-response protein in yeast.

INTRODUCTION

Sphingolipids are ubiquitous constituents of eukaryotic biomembranes. There is now substantial evidence that sphingolipids and their metabolic products are involved in the regulation of cell growth, differentiation and programmed cell death via their participation as second messengers in specific signal-transduction pathways [1,2].

Sphingosine 1-phosphate (S-1-P), which results from the phosphorylation of sphingosine by a specific sphingosine kinase (SK), is the catabolic product of all higher-order sphingolipids. S-1-P has been demonstrated to be involved in the regulation of mammalian cell proliferation, apoptosis, mobility and tumour-cell invasiveness. Although signalling via a specific G-protein-coupled receptor has been implicated in mediating S-1-P-induced biology [3,4], considerable controversy remains regarding which effects attributable to S-1-P are a result of intracellular accumulation, and which are due to extracellular receptor-mediated events. As would be expected for a potent second messenger, S-1-P is maintained at very low baseline intracellular levels [5]. The mechanisms by which the cell maintains S-1-P levels are not known, but could involve regulation of S-1-P synthesis by ceramidases and/or SK, or by regulation of its degradation by sphingosine phosphate lyase (SPL) and/or sphingosine phosphate phosphatases.

SK activity has been demonstrated in tissues from a variety of species, including human and porcine platelets [6], bovine brain [7] and kidney [8], rat liver, *Tetrahymena pyriformis* and the yeast *Hansenula ciferrii* [9]. SK from all sources requires both a bivalent cation (magnesium, calcium or manganese) and a phosphate donor (ATP) for the phosphorylation of the hydroxy group on the first carbon of sphingosine. Maximal activity from all sources evaluated to date requires a fixed ratio of magnesium-to-ATP of 5:1, and a pH of 7.2–7.5. SK is found in the cytoplasm of human and porcine platelets [6], whereas it is associated with membranes in rat brain [10] and several other tissues. D-*erythro*-Sphingosine, the naturally occurring isomer of sphingosine and most abundant sphingoid base in many mammalian cells, serves as a substrate for SK from all sources. Stereospecificity for the *erythro* conformation has been demonstrated in mixed micellar assays using human platelet- and rat brain-derived enzymes. A variety of long-chain bases can also serve as substrates for SK, including *erythro*-dihydrosphingosine and phytosphingosine. SK activity increases with the carbon chain length of a D-*erythro*-dihydrosphingosine substrate. Stimulation of Swiss 3T3 cells with some inducers of proliferation (fetal calf serum or platelet-derived growth factor) results in an increase in both sphingosine levels and SK activity, indicating that the enzyme activity is regulated in some manner [11]. The mechanism by which SK is stimulated by growth factors is not understood. Additional mitogens, such as the β subunit of the cholera toxin and PMA, also significantly increase enzyme activity. Stimulation is specific, as demonstrated by the fact that several other mitogens (epidermal growth factor, bombesin and insulin) have little effect on SK activity. SK has not been purified to homogeneity, and the gene encoding SK has not been isolated from any source.

Sphingolipids also exist in the yeast *Saccharomyces cerevisiae*, where they provide a vital but unidentified function, possibly involving mediation of stress responses [12–14]. Several aspects of sphingolipid metabolism and signal transduction appear to be conserved in *S. cerevisiae* [15], therefore yeast may provide a useful model in which to dissect the components of sphingolipid signalling. We recently used this system to isolate the *S. cerevisiae* SPL gene, *DPL1* (formerly *BST1*; dihydrosphingosine-1-phosphate lyase 1), providing the first SPL nucleotide sequence from any organism [16]. The *dpl1*Δ mutants accumulate S-1-P, and are extremely sensitive to treatment with D-*erythro*-sphingosine,

Abbreviations used: S-1-P, sphingosine-1-phosphate; SK, sphingosine kinase; SPL, sphingosine-1-phosphate lyase; *DPL1*, dihydrosphingosine-1-phosphate lyase 1; NP40, Nonidet P40.

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owing to a failure to degrade S-1-P to inactive metabolites. This accumulation of S-1-P suggests that *S. cerevisiae* has SK activity, although SK activity in *S. cerevisiae* has not previously been described. The *dpl1*Δ mutants demonstrate increased tolerance to stress conditions [17], indicating that endogenous phosphorylated sphingoid bases may be involved in the response to stress in yeast.

In this study, we have characterized *S. cerevisiae* SK activity, and have identified features which suggest SK may function as a stress-response protein in yeast. Additionally, mutants demonstrating a loss of SK activity have been isolated utilizing a screen for suppressors of sphingosine sensitivity in a strain in which the *DPL1* gene has been deleted. The phenotype of these SK-deficient mutants supports the hypothesis that SK is involved in mediating responses to stress in yeast. Furthermore, these mutants implicate intracellular phosphorylated sphingoid base accumulation, rather than their presence at extracellular sites, in mediating these responses.

MATERIALS AND METHODS

Materials

Sphingosine, dihydrosphingosine and phytosphingosine were obtained from Sigma (St. Louis, MO, U.S.A.). (D,L)-*threo*-Dihydrosphingosine was obtained from Biomol (Plymouth Meeting, PA, U.S.A.).

Yeast strains and growth conditions

The *S. cerevisiae* strain DG338 (*MAT*α hisΔ*2OO ura3-167 GAL*) was used in all studies [18], with these exceptions: the diploid JK93d strain (*ura3-52 leu2-3,112 his4 trpl rmel*) was used for sporulation; JS35 strain (DG338 *dpl*::*NEO*), SGP3 (*leu2-3,112 trp1 ura3-52 his3 ade8 ras*::*HIS3*) and JS16 (SGP3 *dpl1*::*NEO*) were used for isolation and characterization of spontaneous sphingosine-resistant mutants. JS62 and JS63 are sphingosine-resistant/SK$^-$ mutants of JS35. JS80 is a sphingosine-resistant/SK$^-$ mutant of JS16. Yeast strains were maintained on rich medium [YPD (yeast extract/peptone/dextrose)] or minimal medium [SC (synthetic complete)], as described previously [19]. Where indicated, glucose was not added, and the medium included 3% (v/v) glycerol, 3% (v/v) ethanol or 2% (w/v) potassium acetate. Sporulation medium was made with 10 g of potassium acetate, 1 g of yeast extract and 0.5 g of dextrose per litre.

SK assay

Wild-type or mutant strains of yeast were grown to a D_{600} of 3.0, unless otherwise stated. Cytosolic extracts were prepared by collecting 2×10^9 cells, washing in 10 ml of distilled H_2O and resuspending in 1 ml of lysate buffer [100 mM Mops (pH 7.2)/5% (v/v) glycerol/10 mM $MgCl_2$/5 mM 2-mercaptoethanol]. A 1 M sodium acetate buffer system was used in place of Mops to optimize pH. Glass beads (800 μl) were added, cells were vortex-mixed for 30 s, and then placed on ice for 30 s for 12 rounds. Centrifugation was performed for 10 min at 4 °C (300 ***g***), and the supernatant was then re-centrifuged at 16000 ***g*** for 5 min at 4 °C. The supernatant was frozen on solid CO_2/ethanol, and stored at −80 °C. Protein concentration was estimated using the Bradford method (Bio-Rad). Unless otherwise stated, SK activity was assayed at 43 °C, with 100 μg of cytosolic protein and 3.5 mol% D-*erythro*-sphingosine (1.8 mM) for 20–30 min. A mixed octyl-β-D-glucopyranoside/sphingosine micellar system was used (substrate concentrations were calculated as molar percentages of octyl-β-D-glucopyranoside) with [γ-^{32}P]ATP, and the radiolabelled product, S-1-[^{32}P]P, was converted to caproyl S-1-[^{32}P]P, as described previously [20]. After a series of extractions, 50 μl of the lower phase was transferred to scintillation vials, evaporated to dryness and counted for radioactivity in a Packard Liquid Scintillation Analyser (Downers Grove, IL, U.S.A.) The remainder of the chloroform phase was dried, resuspended in chloroform/methanol (4:1, v/v), separated by TLC and detected by autoradiography. The product *N*-caproyl-S-1-P migrated with an R_F 0.19, as previously described [20], when using Silica-G 0.5-mm plates from Curtin Mathaeson Scientific (Pittsburgh, PA, U.S.A.) Upon subsequent modification of the protocol, Silica-G 0.25-mm plates were used (Analtech, Newark, DE, U.S.A.), and the product migrated with an R_F 0.29. All assays were performed in duplicate.

Extract collection for growth curve, carbon source and sporulation

Growth curves were determined by harvesting cells at various culture densities, as determined spectrophotometrically, followed by extraction, as described above. For carbon source experiments, yeast cells were grown in rich media containing 2% (w/v) glucose to a D_{600} of 1.5, collected by centrifugation, washed with distilled H_2O, collected again by centrifugation, and used to inoculate rich media with either 2% (w/v) glucose or 3% (v/v) ethanol as a sole carbon source. Cells were incubated in the designated media for 3.5 h, harvested, normalized for cell number and used to prepare extracts, as described above. To induce sporulation, cells were grown in rich media to a D_{600} of 3.0, an aliquot of cells was collected, and cytosolic extracts were made for the zero time point, and the remaining cells were collected, washed and used to inoculate sporulation media to an equal attenuance. After every 24 h for 4 days, percentages of tetrads per 300 cells counted (using a haemocytometer) were determined, aliquots of cells were collected and cytosolic extracts were obtained. SK assays were then performed as described above.

Screen for suppressors of *dpl1*Δ

The *dpl1*Δ strains JS35 and JS16 were inoculated to a density of 1×10^4 cells per plate on to agar plates containing 100 μM D-*erythro*-sphingosine and 0.0015% (w/v) Nonidet P40 (NP40) (used as a dispersant). At these concentrations of sphingosine and this cell density, the *dpl1*Δ strains JS35 and JS16 do not grow. Plates were incubated for 3–5 days, and rare spontaneous mutants were isolated, evaluated for sphingosine resistance using the dilutional assay described below, and for SK activity using the method described above.

Sphingosine dilutional assay

Cells were grown to saturation for 2–3 days in liquid cultures. They were then resuspended in minimal medium, placed in the first row of a 96-well plate and diluted serially from 1:2 to 1:4000 across the plate. The cultures were then template-inoculated on to a control plate (rich medium) and a plate containing 50 μM sphingosine and 0.0015% (w/v) NP40. (At this concentration of NP40, no effects on cell viability are observed.) Plates were incubated at 30 °C for 2 days and assessed visually for differences in growth.

S-1-P measurements

Cells were grown to a D_{600} of 3.0 in rich medium, resuspended in minimal medium containing 0.0015% (w/v) NP40 and 100 μM

D-*erythro*-sphingosine, and incubated at 30 °C with shaking for 2 h. After washing twice with media containing no sphingosine, S-1-P was extracted and acylated with [^{3}H]acetic anhydride. The radiolabelled C_2-ceramide-1-phosphate product was isolated by TLC, and measured as described previously [21,22].

Growth curves and heat shock

Growth curves were determined by inoculating rich media with exponentially growing cells to a D_{600} of 2.0, then harvesting samples at various time points and determining D_{600}. For heat-shock experiments, saturated cultures were diluted to 10^4 cells/ml in fresh rich media, and incubated in a 50 °C water bath for various time periods. Before heat shock and during the heat treatment, 100 μl aliquots of cells were removed and immediately diluted to 10^3 cells/ml into rich media at room temperature. Aliquots of 100 μl (approx. 100 cells) were plated on to each of two rich-medium plates. Plates were incubated at 30 °C for 2 days, and then colonies were counted.

RESULTS AND DISCUSSION

Characteristics of SK activity *in vitro* in *S. cerevisiae*

SK activity was evaluated using D-*erythro*-sphingosine and [γ-^{32}P]ATP in a reaction which measures the amount of phosphorylated sphingosine after a secondary conversion to *N*-caproyl-S-1-P. Cytosolic extracts prepared from exponentially growing wild-type *S. cerevisiae* cells contained substrate-dependent SK activity at neutral pH and 37 °C (Figure 1). This activity was also dependent on protein concentration and time (results not shown). There was minimal difference in recovery of *N*-caproyl-S-1-P between cytosolic and whole-cell extracts (results not shown). Thus we inferred that SK activity in *S. cerevisiae* resides primarily in the cytosol, similarly to the SK activities of mammalian sources.

Sphingolipids of mammalian cells contain predominantly sphingosine as the long-chain base, whereas yeast sphingolipids contain predominantly phytosphingosine and, to a lesser extent, dihydrosphingosine. Yeast SK was capable of phosphorylating each of these substrates; the K_m values for sphingosine, dihydrosphingosine and phytosphingosine were 0.2, 0.38 and 1.2 mol % (Figure 2). Similarly to SK from mammalian sources, sphingosine has a higher K_m than dihydrosphingosine, and phytosphingosine is not as active a substrate for yeast SK [9]. (D,L)-*threo*-Dihydrosphingosine is an inhibitor of SK activity from mammalian sources. In *S. cerevisiae*, neither phosphorylation of (D,L)-*threo*-dihydrosphingosine nor inhibition of sphingosine phosphorylation by (D,L)-*threo*-dihydrosphingosine were detectable at dihydrosphingosine concentrations up to 5 mol % (Figure 3). There was a linear increase in K_m and V_{max} with increasing (D,L)-*threo*-dihydrosphingosine. The reasons for this are not obvious, but may involve changes in substrate presentation, as (D,L)-*threo*-dihydrosphingosine may alter the dynamics of the mixed micelle system used in this assay. Lastly, yeast SK activity was not inhibited by S-1-P (results not shown), suggesting a lack of direct end-product inhibition.

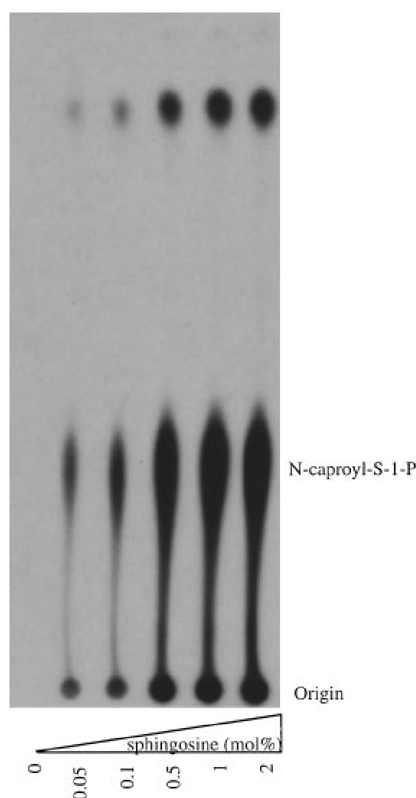

Figure 1 Demonstration of SK activity in cytosolic extracts of S. cerevisiae

Cytosolic extracts were obtained and SK assays were performed as described in the Materials and methods section, with increasing concentrations of sphingosine as substrate (expressed as mol% of octyl-β-D-glucopyranoside). The radiolabelled product S-1-[^{32}P]P is caproylated to generate the reaction product caproyl-S-1-[^{32}P]P. Shown is an autoradiogram of the reaction products recovered by TLC in a solvent system containing chloroform/pyridine/formic acid (60:30:7, by vol.). Other labelled lipids are presumably phospholipids, which can be labelled with the incorporation of ethanolamine phosphate, a catabolic product of S-1-P.

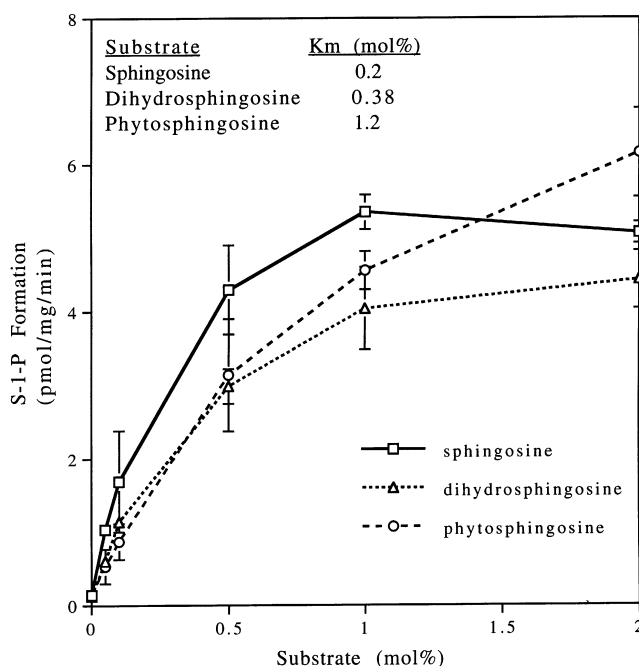

Figure 2 Kinetics of *S. cerevisiae* SK activity using various sphingoid bases as substrates

Shown are substrate concentration plots of SK activity for sphingosine, dihydrosphingosine and phytosphingosine (expressed as mol % of octyl-β-D-glucopyranoside). Each assay was performed three times, and K_m values were calculated using six different programme parameters.

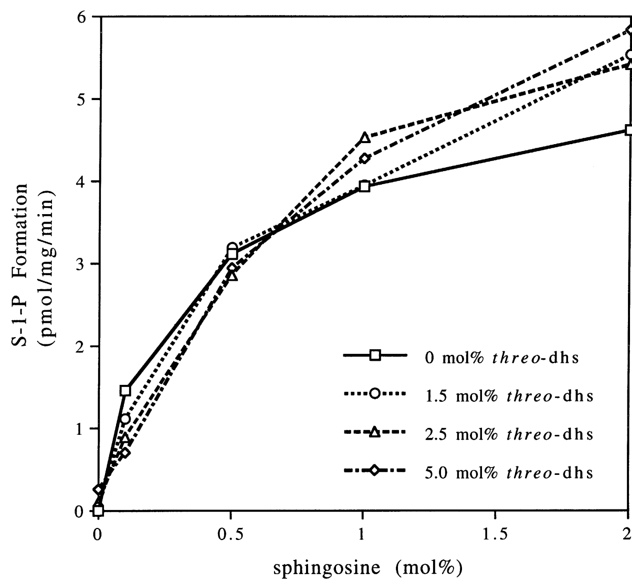

Figure 3 (D,L)-*threo*-Dihydrosphingosine does not inhibit *S. cerevisiae* SK

SK activity was determined as described above, using increasing concentrations of sphingosine substrate and increasing concentrations (from 0 to 5 mol%) of (D,L)-*threo*-dihydrosphingosine (*threo*-dhs). Small differences in activity at low and high sphingosine concentrations were not statistically significant. Data are representative of three experiments.

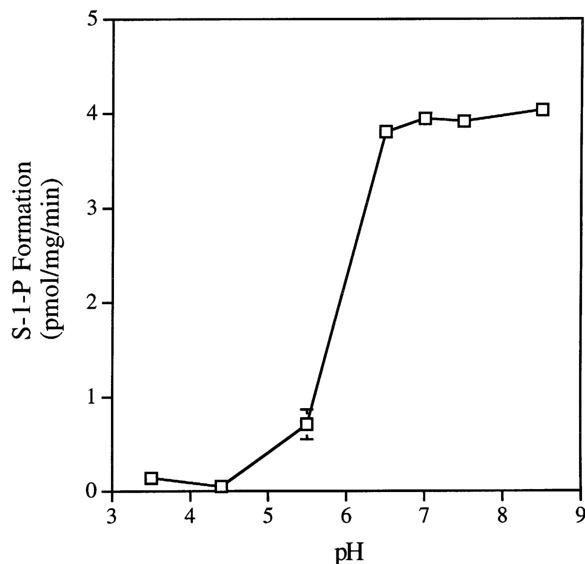

Figure 4 *S. cerevisiae* SK activity is optimal at neutral pH

SK activity was determined at different pH values, using a sodium acetate buffer system, as described in the Materials and methods section. Data are representative of three experiments.

Yeast SK demonstrated a sharp increase in activity above pH 5.5, and remained active at up to pH 8.5, the most alkaline pH examined (Figure 4). The optimal reaction temperature for *S. cerevisiae* SK was 43 °C, and activity remained high even at 47 °C (Figure 5). Because many enzymes are inactivated at these temperatures, we considered this to be a significant observation, consistent with evidence to suggest that sphingolipid metabolites have a role in mediating heat-shock responses in yeast [14].

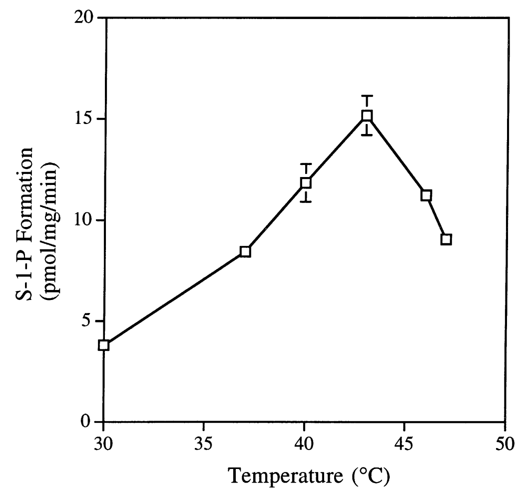

Figure 5 *S. cerevisiae* SK activity is optimal at very high temperatures

SK activity was determined at various temperatures. Data are representative of three experiments.

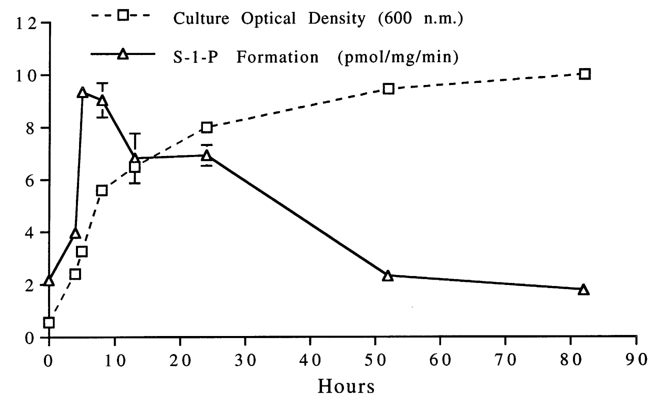

Figure 6 *S. cerevisiae* SK is activated during nutrient-deprivation conditions

Cells were grown in rich media and harvested at different times. The attenuance (*D*) of the culture was measured at 600 nm, and extracts were prepared from these samples and evaluated for SK activity. Data are representative of two experiments.

SK activity response to nutrient conditions in *S. cerevisiae*

Our previous observations suggest a role for phosphorylated sphingoid-base signalling in response to nutrient conditions (D. Gottlieb, W. Heideman and J. Saba, unpublished work). Therefore we were interested in determining whether SK activity responds to changes in nutrient conditions throughout the growth curve. Cultures inoculated into rich media grow exponentially until significant glucose depletion occurs. At the stage where cultures reach a D_{600} of 6, glucose in the media has been depleted to approx. 50 % of starting concentrations, and carbon metabolism begins to switch from the fermentative to the respiratory mode [23]. This point is defined as the diauxic shift. Cultures grow for 20 h or more, utilizing respiratory metabolism during the post-diauxic phase. Eventually, cultures cease to proliferate and enter into G_1 cell-cycle arrest, a period defined as the stationary phase. Cytosolic extracts were prepared from yeast cells harvested at different points in the growth curve, from early

Table 1 *S. cerevisiae* SK activity decreases during sporulation

Yeast cells were induced to sporulate, and cytosolic extracts were obtained from cells on days 0, 1, 2, 3 and 4 during sporulation. Cells grown in rich media, before transfer to sporulation media, were used for the day zero time point. Extracts were prepared from these samples and evaluated for SK activity. The percentages of sporulation were determined at each time point.

Days in sporulation media	S-1-P Formation (pmol/mg per min)	% Sporulated
0	13.19 ± 0.34	0
1	7.41 ± 0.91	14.6
2	6.15 ± 0.13	22.0
3	6.09 ± 0.48	59.2
4	4.01 ± 0.29	62.4

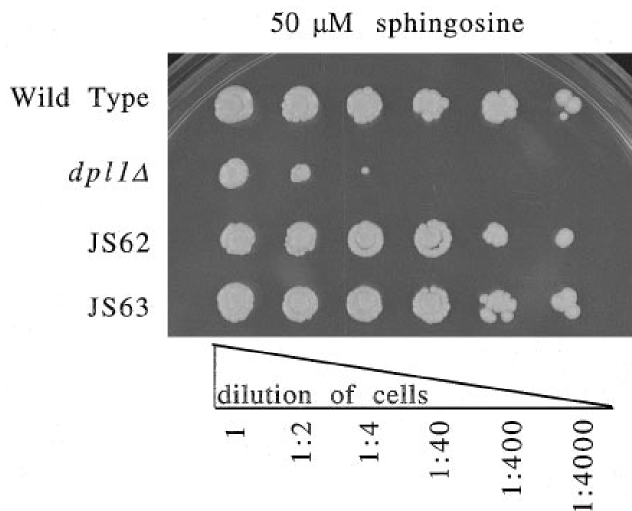

Figure 7 Growth of wild-type, *dpl1*Δ and spontaneous mutants on sphingosine-enriched media

Wild-type (DG338), *dpl1*Δ (JS35) and mutant clones (JS62 and JS63) were grown to saturation, and an equal number of cells were collected and used for serial dilutions, ranging from concentrated (1) to most dilute (1 : 4000). Cells were then transferred to plates containing 50 μM sphingosine.

exponential to stationary phases (Figure 6). SK activity was low during early exponential phase, peaked immediately before the diauxic shift, and decreased during the post-diauxic shift and throughout the approach to stationary phase. Thus we conclude that *S. cerevisiae* SK is responsive to nutrient supply, and may be involved in the shift from fermentation to respiration. To characterize SK response to a non-fermentable carbon source, we measured SK activity in yeast cells grown in glucose or ethanol as the sole carbon source. SK activity from cells grown in ethanol was 64 % that of the activity found in cells grown in glucose. This finding is consistent with the decrease in SK activity after the shift to respiration in the post-diauxic phase of growth (Figure 6). Lastly, we examined yeast SK activity in response to sporulation conditions, which provide the stress of nitrogen deprivation and a poor non-fermentable carbon source. Cytosolic extracts were obtained from a diploid yeast strain that was induced to sporulate over 4 days (Table 1). SK activity dropped significantly on the first day of sporulation, and remained low as the percentage of sporulated cells increased to 62.4 % on day 4. These results indicate that yeast SK activity diminishes during sporulation.

Table 2 S-1-P levels in wild-type, *dpl1*Δ, and sphingosine-resistant strains

Wild-type (DG338), *dpl1*Δ (JS35) and putative SK mutant strains (JS62 and JS63) were incubated in sphingosine. S-1-P was extracted, acylated with [^{3}H]acetic anhydride, and the radiolabelled C_2-ceramide-1-P product was isolated by TLC and quantified by PhosphorImager. Experiments were performed in duplicate.

Strain	$10^{-3} \times$ S-1-P levels (PhosphorImager counts)
Wild-type	2.78 ± 1.84
*dpl1*Δ	9.82 ± 9.94
JS62	0.50 ± 0.17
JS63	0.50 ± 0.58

Isolation of SK-deficient mutants

Sphingoid long-chain bases are metabolized by phosphorylation (catalysed by SK), followed by cleavage of the phosphorylated product at the C^2–C^3 bond by SPL. The *DPL1* gene of *S. cerevisiae* has recently been cloned and identified as encoding the first known SPL. The yeast *dpl1*Δ strain is extremely sensitive to sphingosine, owing to its inability to degrade S-1-P. Exposure to low concentrations of D-*erythro*-sphingosine results in accumulation of S-1-P, which is toxic to the yeast cell. This phenotype can be utilized to isolate mutants that cannot accumulate S-1-P, and therefore are resistant to D-*erythro*-sphingosine. Mutations in the SK gene leading to diminished SK activity would reduce or eliminate production of S-1-P. Therefore, we selected for spontaneous sphingosine-resistant suppressors of *dpl1*Δ, in an attempt to isolate SK-deficient mutants.

To ensure that deletion of *DPL1* has no effect on SK activity, we first compared the SK activity present in wild-type and *dpl1*Δ strains. We observed no significant differences in the SK activity of these two strains, indicating that *S. cerevisiae* SK is not subject to end-product inhibition *in vivo*, and that identification of SK mutants in the designed genetic screen should be possible. We subjected a population of *dpl1*Δ cells to a selection on D-*erythro*-sphingosine-enriched plates in order to isolate spontaneous suppressors of sphingosine sensitivity.

Utilizing this approach, we isolated a number of suppressor mutants with significantly greater resistance to the growth-inhibitory effects of sphingosine than the original *dpl1*Δ cells (Figure 7). Extracts from each mutant isolated in the screen demonstrated either diminished or absent SK activity using the standard assay under optimal conditions, and within the linear range for protein concentration. To determine whether these putative SK mutants were capable of phosphorylating a sphingoid base substrate, we incubated several clones with 100 μM D-*erythro*-sphingosine and performed S-1-P measurements. The strain in which the selection was performed (*dpl1*Δ) is unable to catabolize S-1-P, and therefore S-1-P was accumulated when exposed to exogenous sphingosine. Sphingosine-resistant mutants of *dpl1*Δ synthesized no detectable S-1-P (Table 2), indicating that SK activity was, in fact, absent.

Phenotype of a sphingosine-resistant mutant

Our previous observations in the *dpl1*Δ strain suggest that phosphorylated sphingoid bases may play a role in the response to nutrient deprivation. This led us to evaluate SK-deficient mutants for abnormalities in growth. Interestingly, we observed a complete cessation of proliferation in the SK-deficient mutant strain JS80 as cells reached the diauxic shift (Figure 8). At this time, wild-type cells shift from fermentative to respiratory metabolism, undergo a brief cell-cycle arrest and then resume

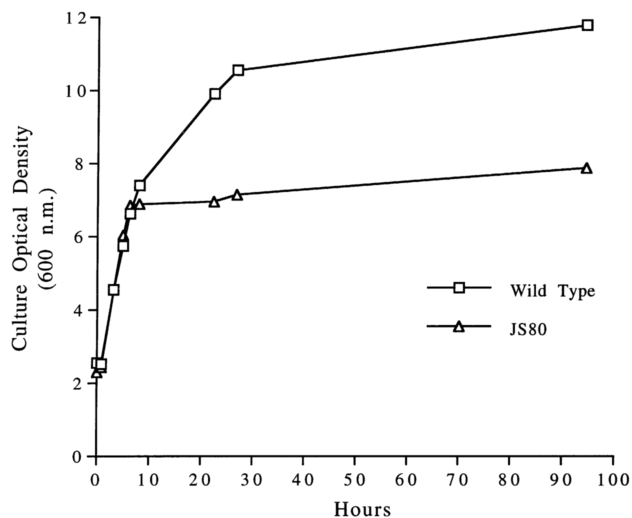

Figure 8 Growth curves of *S. cerevisiae* wild-type and sphingosine-resistant mutant JS80

Exponential-phase wild-type or JS80 cells were used to inoculate rich media. The culture attenuance at 600 nm was determined at various time points throughout the growth curve.

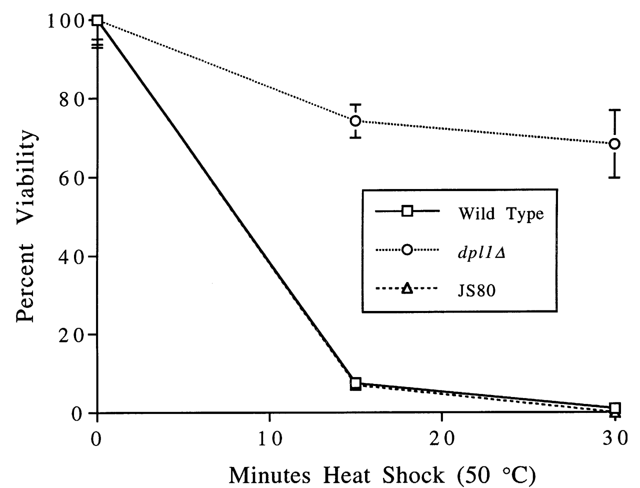

Figure 10 Viability of *S. cerevisiae* wild-type, *dpl1*Δ and spontaneous mutant JS80 cells after heat shock

Wild-type (SGP3), *dpl1*Δ(JS16), and JS80 cells were grown to saturation and incubated at 50 °C for 0–30 min. Viability was determined, and the number of cells viable at zero time is set to 100%. This is representative of three experiments.

growth at a slower rate. Simultaneously, *dpl1*Δ cells demonstrate enhanced proliferation in comparison with wild-type cells. These observations suggest that SK-deficient mutants are unable to respire. To investigate this further, we evaluated the growth of wild-type (SGP3), *dpl1*Δ (JS16) and JS80 strains on media containing different carbon sources (Figure 9). The SK-deficient mutant was unable to grow on glycerol, ethanol or acetate as a sole carbon source, in either rich or minimal medium. These results indicate that the SK-deficient mutant is unable to utilize non-fermentable carbon sources. The characteristics of the JS80 mutant are representative of numerous spontaneous SK^- mutants isolated on the basis of their resistance to sphingosine. Taken together with our previous observations in the *dpl1*Δ strain, these studies suggest a role for phosphorylated sphingoid bases in mediating the shift from fermentative to respiratory metabolism.

Our previous finding of increased heat resistance in the *dpl1*Δ mutant suggests a role for phosphorylated sphingoid bases in response to heat shock. We were therefore interested in determining whether the heat-shock resistance of the *dpl1*Δ strain would be abrogated by loss of SK activity. To investigate this, wild-type, *dpl1*Δ and JS80 strains were grown to saturation, incubated at 50 °C for 0 to 30 min, and evaluated for viability (Figure 10). We observed the expected heat-shock resistance of the *dpl1*Δ strain, and a reversal of this phenotype in the JS80 strain. These results indicate that phosphorylated sphingoid base accumulation is directly responsible for the heat-shock resistance of the *dpl1*Δ strain, and support a role for phosphorylated sphingoid bases in mediating the heat-shock response in yeast.

CONCLUSION

These studies demonstrate the presence of SK activity in *S. cerevisiae*. Whereas the K_m values and optimal pH range for

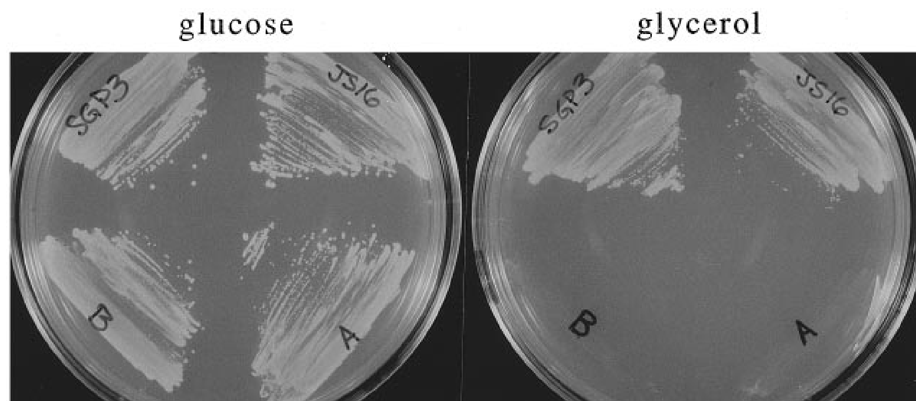

Figure 9 Growth of wild-type, *dpl1*Δ and spontaneous mutant JS80 on plates containing either glucose or glycerol

Yeast cells were inoculated on to synthetic media containing 2% (w/v) glucose (left) or 3% (v/v) glycerol (right). Strains used were wild-type (SGP3, upper left), *dpl1*Δ (JS16, upper right) and a duplicate of JS80 (**A** and **B**, lower left and right). Plates were incubated at 30 °C for 3 days and photographs were taken. Plates incubated for up to 10 days showed the same pattern of growth.

S. cerevisiae SK are consistent with reports of SK activity from other species, the optimal temperature range for yeast SK is high, between 40 and 50 °C. In fact, SK activity at 47 °C is more than twice as high as that at 30 °C, the optimal growth temperature for *S. cerevisiae*. This suggests that yeast SK is highly tolerant to heat, and could act as a heat-shock protein. Yeast SK activity appears highest just before the diauxic shift, at a time when glucose is diminishing and yeast cells initiate a shift from fermentation to respiration. SK activity decreases throughout the post-diauxic phase, in the presence of a non-fermentable carbon source, and during sporulation. These results suggest that SK activity is responsive to nutrient conditions, and that phosphorylated sphingoid bases may be involved in mediating the shift from fermentation to respiration.

We have demonstrated the utility of the yeast SK assay in evaluation of sphingosine-resistant mutants. We conclude that these strains contain mutations within genes that are either directly or indirectly responsible for maintaining SK activity. Identification of the sites of mutation by genetic complementation is currently underway. The failure of SK-deficient mutants to pass through the diauxic shift and successfully initiate growth supported by respiratory metabolism corroborates our previous findings, and further supports a role for phosphorylated sphingoid bases in the initiation of respiration. Importantly, the observation that loss of SK activity results in a reversal of the characteristic resistance of the *dpl1*Δ strain to both nutrient deprivation and heat shock suggests that intracellular phosphorylated sphingoid base accumulation is directly responsible for these characteristics. Although we cannot rule out the possibility that phosphorylated sphingoid bases are secreted or transported outside the cell to act upon extracellular receptors of neighbouring cells, no evidence exists to support this, and our inability to enhance growth rate using exogenous dihydro-S-1-P suggests this is not the case.

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