Inhibition of Amino Acid Transport by Sphingoid Long Chain Bases in \textit{Saccharomyces cerevisiae}*

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Marek S. Skrzypek, M. Marek Nagiec, Robert L. Lester, and Robert C. Dickson‡

*From the Department of Biochemistry and the Lucille P. Markey Cancer Center, University of Kentucky Medical Center, Lexington, Kentucky 40536-0084

Sphingoid long chain bases have many effects on cells including inhibition or stimulation of growth. The physiological significance of these effects is unknown in most cases. To begin to understand how these compounds inhibit growth, we have studied \textit{Saccharomyces cerevisiae} cells. Growth of tryptophan (Trp) auxotrophs was more strongly inhibited by phytosphingosine (PHS) than was growth of Trp strains, suggesting that PHS diminishes tryptophan uptake and starves cells for this amino acid. This hypothesis is supported by data showing that growth inhibition is relieved by increasing concentrations of tryptophan in the culture medium and by multiple copies of the TAT2 gene, encoding a high affinity tryptophan transporter. Measurement of tryptophan uptake shows that it is inhibited by PHS. Finally, PHS treatment induces the general control response, indicating starvation for amino acids. Multiple copies of TAT2 do not protect cells against two other cationic lipids, stearylamine, and sphingosine, indicating that the effect of PHS on tryptophan utilization is specific. Other data demonstrate that PHS reduces uptake of leucine, histidine, and proline by specific transporters. Our data suggest that PHS targets proteins in the amino acid transporter family but not other distinctly related membrane transporters, including those necessary for uptake of adenine and uracil.

The long chain base component of sphingolipids inhibits cell growth under many conditions and is cytotoxic to some cell types, but the mechanisms underlying these phenomena are not well characterized. Interest in the effect of long chain bases on cells was stimulated by the observation that they are potent inhibitors of protein kinase C (1–3). Since these initial studies, there has been a growing number of reports showing effects of long chain bases on a variety of cells (reviewed in Refs. 4 and 5). Most of these studies have been performed on cultured mammalian cells, and it is not clear if the observed effects of long chain bases actually cause growth inhibition or whether some secondary effect inhibits growth. To try and understand how long chain bases inhibit growth, we have examined the effect of long chain bases on \textit{Saccharomyces cerevisiae} cells.

Sphingolipids are an essential component of wild type \textit{S. cerevisiae} cells (6). Synthesis of the long chain base component of sphingolipids is similar to that in mammals and begins with the condensation of serine and palmitoyl-CoA to yield 3-ketosphinganine (for review, see Ref. 7). The 3-ketosphinganine is converted to the long chain base sphinganine (dihydrosphingosine) which is N-fatty-acylated to yield dihydroceramide. Dehydrogenation of dihydroceramide in animals yields ceramide containing the long chain base sphingosine, which is rapidly converted to sphingolipids by the addition of polar components to the 1-hydroxyl group. The predominant ceramides in fungi and plants contain N-α-hydroxyfattyacylphosphosphingosine (6). Phytosphingosine (PHS) lacks the 4,5-double bond found in sphingosine and has instead an hydroxyl group at the 4-position. In \textit{S. cerevisiae} and other fungi, the 1-hydroxyl of phytoceramide is modified by addition of myo-inositol phosphate to form inositol phosphoceramide, which is then mannosylated to yield mannose-inositol phosphoceramide. The final step in \textit{S. cerevisiae} sphingolipid synthesis is the addition of inositol phosphate to mannose-inositol phosphoceramide to yield the major sphingolipid mannose-(inositol-P)$_3$ ceramide (8–10).

When grown in complex medium having a non-inhibitory concentration of long chain base, \textit{S. cerevisiae} cells are able to take up the long chain base, by an unknown mechanism that requires the multimembrane-spanning Lcb3 protein (11), and incorporate it into ceramide and other sphingolipids (12). Surprisingly, even the non-biological \textit{three} isomer of sphinganine can be incorporated into sphingolipids (12). When present in high enough concentrations, most long chain bases will inhibit growth of \textit{S. cerevisiae} cells. However, growth inhibition is only seen if the culture density is kept very low, for example, less than $10^7$ cells/ml (13).

After initiating these studies, we observed that growth is more strongly inhibited by long chain bases in strains requiring amino acids (auxotrophic strains), particularly tryptophan and leucine. Amino acid transport in \textit{S. cerevisiae} is a complex and highly regulated process because amino acids serve not only as building blocks for protein synthesis, but also as a source of nitrogen when a preferred nitrogen source, such as ammonia, glutamine, or asparagine, is unavailable. In the absence of a preferred nitrogen source, for example, when proline or urea are the primary nitrogen source, amino acids are transported by both specific amino acid permeases and by the general amino acid permease (Gap1p; Ref. 14). Gap1p transports all natural L-amino acids found in proteins and also ornithine, citrulline, and several d-amino acids and toxic amino acid analogs (15).

Gap1p activity is repressed when a preferred nitrogen source is available, a phenomenon referred to as nitrogen catabolite repression (16). Repression of Gap1p activity occurs at several levels by complex regulatory mechanisms (14, 17, 18). In con-
trast, the activity of some, but not all, specific amino acid permeases is constitutive and not repressed by preferred nitrogen sources (19).

We demonstrate here that PHS blocks transport of tryptophan, leucine, histidine, and proline by their specific amino acid transporters. Inhibition of amino acid transport explains why PHS blocks growth of auxotrophic *S. cerevisiae* strains and why prototrophic strains are resistant to the drug.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Culture Conditions**—Strains used in this work are shown in Table I. Plasmids pAS5 and pAS6 carried the *TAT1* and *TAT2* gene, respectively, on YEp351 and were provided by Michael Hall (Biozentrum, Basel, Switzerland).

Yeast were grown at 30 °C in complex medium (PYED) buffered to pH 5.0 as described (20) or in defined medium (SD) containing 0.17% yeast nitrogen base (U. S. Biological, Swampscott, MA), 2% glucose, and 0.5% ammonium sulfate. Proline (1 mg/ml) was used as a nitrogen source in place of ammonium sulfate where indicated. SD complete medium was supplemented with adenine sulfate, uracil, L-leucine, L-threonine, L-lysine, L-valine, and L-threonine (all at 20 mg/ml); L-isoleucine (30 mg/liter); L-valine (150 mg/liter); and L-threonine (1 mg/ml). Yeast were grown in SD medium (minus uracil and histidine but containing 0.05% Tergitol as a carrier for PHS) and were transformed with both pRS313(*HIS3*) and pRS315(*LEU2*), YCp405(*LYS2*), and pRS316(*URA3* *ADE2*) (this study).

**RESULTS**

**Tryptophan Auxotrophs Are Sensitive to PHS**—An initial screening of *S. cerevisiae* strains for inhibition of growth by PHS revealed differences in sensitivity. Strains such as YPH250 were inhibited (PHS-sensitive, PHS^S^), whereas other strains such as SJ19 were not (PHS-resistant, PHS^R^). Segregation analysis demonstrated a genetic basis for this difference in sensitivity; five four-spored tetrads gave 2:2 segregation for the PHS^R^:PHS^S^ phenotypes, showing that the phenotypes are due to a single, nuclear gene. Since the PHS^R^ and Trp^−^ phenotypes co-segregated, the gene responsible for the PHS^R^ phenotype must be *TRP1* or a closely linked gene. The PHS^R^ phenotype was dominant to the PHS^S^ phenotype.

TRP1 is most likely the gene responsible for the PHS^R^ phenotype because transformation of strain YPH250 with the centromeric plasmid pRS314 (*TRP1*) gave PHS^S^ cells. Since pRS314 carries a portion of the *GAL3* gene that is linked to *TRP1*, we verified that cells transformed with a plasmid carrying *GAL3* remained sensitive to PHS. We conclude from these data that tryptophan auxotrophs (Trp^−^) of *S. cerevisiae* are more sensitive to growth inhibition by PHS than are tryptophan prototrophs (Trp^+^).

To quantify the difference in PHS sensitivity between Trp^+^ and Trp^−^ cells and to determine if sensitivity was due to other nutrient requirements, we compared the PHS sensitivity of prototrophic cells (strain YPH-A, YPH252 with all of its nutrient requirements complemented by plasmid-borne genes) to isogenic cells that required only His, Leu, Trp or Ura and Ade (YPH250 transformed with various combinations of plasmids, see Table I). Growth of prototrophic YPH-A cells was only partially inhibited at the highest PHS concentration (PHS is insoluble at higher concentrations), and we estimate, from the apparent midpoint of the inhibition curves, that these cells are at least 10-fold more resistant to PHS inhibition than are the Trp^−^ cells (Fig. 1). His^−^, Leu^−^, and Ura^−^ Ade^−^ cells are as resistant to growth inhibition by PHS as are prototrophic cells (Fig. 1). Similar trends were seen when erythro-dihydrosphingosine or threo-dihydrosphingosine were used in place of PHS (data not shown). Growth inhibition was not observed with the...
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FIG. 1. Growth inhibition by PHS in complex (PYED) medium. Growth inhibition was measured after 24 h of incubation at 30 °C. Results are expressed as a percentage of the cell density reached by the culture lacking PHS. Data are the means ± S.D. from two independent experiments, each done in triplicate. Strains are described in Table I.

FIG. 2. Tryptophan reverses growth inhibition by PHS in complex medium. YPH-D (Trp⁺) cells were grown for 24 h in PYED medium containing the indicated amount of tryptophan. Results are shown as a percentage of the cell density reached by the culture lacking PHS. Data points are means ± S.D. from one experiment done in triplicate.

Water-soluble ceramide mimic C₂-ceramide (N-acetylphosphosine, up to 50 μM), as has been reported by some investigators (27, 28) but not by others (13).

We conclude from the data presented in this section that PHS and dihydrosphingosine inhibit growth of YPH252 cells grown in complex medium (PYED) by blocking tryptophan utilization.

Tryptophan and Enhanced Tryptophan Transport Protect Cells from PHS Inhibition—Growth inhibition by PHS is most likely due to inhibition of tryptophan transport into cells and consequent starvation for this amino acid. This hypothesis predicts that growth inhibition by PHS should be alleviated by increasing the concentration of tryptophan in the culture medium. This prediction was verified (Fig. 2).

Another prediction of this hypothesis is that overexpression of tryptophan transporters should increase resistance to PHS. The Tat2 protein is known to be a high affinity tryptophan transporter, whereas the Tat1 protein is a low affinity tryptophan transporter (29). YPH252 cells transformed with a multicopy vector carrying TAT2 were found to be more resistant to PHS growth inhibition than were cells transformed with the vector alone, whereas cells transformed with a multicopy vector carrying TAT1 showed PHS resistance between that conferred by multiple copies of TAT2 and that conferred by the vector alone (Fig. 3A). These data support the hypothesis that PHS inhibits tryptophan transport.

To determine if PHS was inhibiting growth and tryptophan utilization in a biospecific manner, we compared the effect of PHS with stearylamine, a long chain amine, and sphingosine, the long chain base found in mammals. Sphingosine was a more potent growth inhibitor (Fig. 3C) than PHS (Fig. 3A) which in turn was more potent than stearylamine (Fig. 3B). However, the most significant difference between these three cationic lipids was their response to multiple copies of TAT1 or TAT2. Resistance to stearylamine (Fig. 3B) or sphingosine (Fig. 3C) was only increased 2–3-fold by multiple copies of either gene. In contrast, TAT2 increased resistance to PHS more than 50-fold, whereas TAT1 increased resistance at least 10-fold (Fig. 3A). The ability of multiple copies TAT1 or TAT2 to protect cells against growth inhibition by PHS, but not against inhibition by stearylamine or sphingosine, shows that PHS specifically interferes with tryptophan utilization whereas the other two cationic lipids do not. They must interfere with some other cellular process.

Tryptophan Accumulation Is Inhibited by PHS—To determine directly if PHS inhibits tryptophan accumulation, the rate of uptake of radioactive tryptophan by wild type and mutant strains defective in tat1, tat2, or both genes was determined at low (7–9 μM Trp) and high (70–90 μM Trp) tryptophan concentrations in the presence or absence of PHS. Tryptophan accumulation was inhibited 60–70% at a low tryptophan concentration and about 50% at a high concentration in the presence of 100 μM PHS (Fig. 4A).

Tryptophan uptake by the Tat2 transporter was inhibited 60–70% in the presence of 100 μM PHS at both low and high tryptophan levels (Fig. 4B). Uptake was primarily due to the Tat2 protein (Fig. 4B), since tat2 mutant cells accumulated only a small amount of tryptophan (Fig. 4C). However, some uptake was mediated by the Tat1 protein because the tat1 tat2 double mutant cells showed almost no accumulation compared with the tat2 mutant cells (Fig. 4C), as reported previously (29).

We conclude from these data that PHS inhibits tryptophan accumulation and that accumulation is mediated primarily by the Tat2 transporter under the conditions of these experiments (cells pregrown in complex medium).

The Nitrogen Source Affects PHS Sensitivity—Nutrient transport, particularly amino acid transport, across the plasma membrane is known to be affected by the nitrogen source present in the culture medium (19). In complex culture medium like the PYED medium used here, amino acids serve in part as a source of nitrogen because other nitrogen sources are limiting. Under these circumstances amino acid transport is mediated by both specific amino acid permeases, such as Tat2p, and also by the general amino acid permease (Gap1p). Thus, it is possible that PHS may be inhibiting other specific amino acid transporters but inhibition would not be seen because the amino acid would be transported also by Gap1p and its transport would not become limiting for growth. In addition, the concentration of amino acids in PYED medium is not defined and the concentration might be high enough to overcome the growth inhibiting effect of PHS.

The effect of the nitrogen source on PHS sensitivity was examined first using defined medium (SD) containing ammonium ions as the nitrogen source. In this growth medium, amino acid uptake is thought to occur through specific transporters and not through Gap1p whose synthesis is repressed by ammonium ions (16). Growth of prototrophic YPH-A cells is barely inhibited under these conditions of nitrogen catabolite repression (Fig. 5A), even at the highest possible PHS concentration, unlike the case in PYED medium (nitrogen depressing conditions) where some inhibition of growth is seen at high PHS concentrations (Fig. 1). These data further support the hypothesis that PHS inhibits growth by blocking the activity of
specific amino acid transporters.

Transport of specific nutrients into YPH252 cells was examined by using cells transformed with plasmids so that only one or two nutrients were required. Cells requiring leucine are growth-inhibited by PHS just like the control YPH252 cells that have multiple growth requirements. Thus, the leucine-specific transporters are inhibited by PHS (Fig. 5A).

In contrast to the results found using PYED medium (nitrogen-derepressing conditions, Fig. 1), cells requiring tryptophan are only slightly inhibited for growth at the highest concentration of PHS when grown in defined medium containing ammonium ions (nitrogen-repressing conditions, Fig. 5A). PHS did not inhibit under these conditions because the high concentration (98 μM, 20 μg/ml) of tryptophan in the medium partly overcomes the inhibitory effect of PHS (the concentration of tryptophan in PYED medium is unknown because it is partially destroyed during autoclaving). Cells requiring histidine are slightly more sensitive to growth inhibition by PHS than are prototrophic cells, indicating some inhibition of histidine transport. Cells requiring uracil plus adenine are as resistant to PHS inhibition as the control prototrophic strain YPH-A (Fig. 5A). Therefore, transport of neither nutrient is inhibited by PHS.
The PHS sensitivity of cells was examined next under nitrogen-derepressing conditions by using defined medium having proline as the sole nitrogen source. Under these conditions, proline is transported by the ammonia-repressible proline permease (Put4; Ref. 30), whereas other amino acids are transported by the Gap1p ammonia-repressible permease, which has a much higher transport capacity than the constitutively expressed specific amino acid permeases (31). If PHS inhibits Gap1p permease activity, stronger growth inhibition should be seen in medium containing proline than in medium containing ammonium ions. This prediction was verified for auxotrophic strains requiring one amino acid: His, Trp, or Leu (compare Fig. 5A (ammonia) with Fig. 5B (proline)). The inhibitory effect for each nutrient is nearly additive because the multiple auxotrophic strain YPH252 is more strongly inhibited than are the single-amino-acid-requiring strains (Fig. 5B).

Growth of prototrophic YPH-A and YPH-E cells requiring uracil and adenine was slightly more inhibited by high concentrations of PHS when proline (Fig. 5B) rather than ammonium ions were the nitrogen source (Fig. 5A). The most likely reason for this difference is that PHS inhibits Put4 transport activity in the proline-containing, non-nitrogen-repressing growth conditions (Figs. 1 and 5B), indicating that PHS does not affect these transporters under nitrogen-repressing (ammonia ions in the culture medium) conditions, thus limiting nitrogen availability and reducing the growth yield.

To determine if PHS treatment kills cells, viability was measured by determining colony formation. YPH252 cells transformed with the YEp34 and grown in defined medium lacking leucine were about 80% viable after 6 h of treatment with 50 μM PHS and 40% viable after 48 h of treatment, showing that PHS does not rapidly kill cells.

**PHS Induces the General Control Response—Starvation of Yeast Cells for Amino Acids Induces a Signaling Pathway, the General Control Response, which Increases Translation of GCN4 mRNA.** The resulting Gcn4 protein activates transcription of genes encoding the biosynthetic enzymes necessary for synthesis of many amino acids (32). If PHS is inhibiting amino acid uptake, then PHS treatment should induce the general control response. Induction of the general control response was quantified by measuring β-galactosidase activity in YPH252 cells transformed with a reporter plasmid containing the GCN4 promoter fused to the lacZ gene (plasmid pB180; Ref. 23).

PHS treatment caused a 2.2-fold increase in β-galactosidase activity relative to untreated cells, and this increase is about the same as the 2.3-fold increase seen in cells treated with 3-amino triazole (Table II), an agent known to induce the general control response (23). In control experiments, used to normalize β-galactosidase values (Table II) as described previously (21), PHS did not induce expression of a GCN4-lacZ reporter gene, carried on pB277 (23), lacking the four open reading frames necessary for response to amino acid starvation. We conclude from these data that PHS treatment activates the general control response. These results further support the hypothesis that PHS blocks amino acid uptake.

**DISCUSSION**

The studies reported here demonstrate that growth inhibition of *S. cerevisiae* cells by the sphingoid long chain base PHS is due to inhibition of amino acid transporters and subsequent starvation for amino acids. Most data are for tryptophan transport by the Tat2 transporter, other data show that PHS reduces uptake of leucine, histidine, and proline. No interference with uracil or adenine uptake was observed.

Inhibition of tryptophan uptake by PHS was initially indicated by segregation analysis, which revealed that the sensitive/resistance phenotype was due to a single, nuclear gene and that the resistance phenotype was tightly linked to the TRP1 locus. Because *Trp*<sup>+</sup> cells such as SJ19, YPH-A (Fig. 1) or YPH250 transformed with a plasmid (pRS314) carrying the *TRP1* gene were resistant, whereas *Trp*<sup>−</sup> cells such as YPH-D (Fig. 1) were sensitive to growth inhibition by PHS, we hypothesized that tryptophan uptake was impaired by PHS. Several types of data support this hypothesis. First, increasing concentrations of tryptophan in the culture medium reduce growth inhibition of the *Trp*<sup>−</sup> strain YPH-D by PHS (Fig. 2). Second, growth inhibition by PHS is greatly reduced by multiple copies of *TAT2*, encoding a high affinity tryptophan transporter, and to a lesser extent by multiple copies of the *TAT1*, encoding the low affinity tryptophan transporter (Fig. 3A). Third, PHS treatment diminishes the rate of tryptophan uptake. In PYED medium, most of this uptake is mediated by the Tat2 transporter (Fig. 4). Finally, the general control response is induced by PHS treatment (Table II), indicating that cells are being starved for one or more amino acids.

How might PHS affect Tat2 transport activity? Our data are not consistent with PHS directly inhibiting activity because the rate of tryptophan transport is normal for about 10 min and then it tapers off (Fig. 4). These kinetics are consistent with PHS activating a signal transduction pathway that down-regulates Tat2 activity. PHS is not disrupting the proton gradient necessary for tryptophan transport, because transport of other nutrients, such as uracil, adenine, and vitamins that require a proton gradient, is not blocked.

The Tat2 protein is a member of the major facilitator superfamily (33) whose members contain 12 membrane-spanning α-helices, a hydrophilic N and C terminus, and 500–600 amino acids. These proteins lack the nucleotide-binding domain typical of ABC transporters and the conserved amino acids characteristic of the catalytic site in P-type ATPases. In *S. cerevisiae*, the Tat2, Gap1p, Tat1, and other amino acid transporters belong to a family of closely related proteins that is distinct from the uracil/allantoin transporters and from the purine transporter (19, 34, 35). PHS did not inhibit uracil or adenine transport under nitrogen-repressing (ammonia ions in the culture medium), partially nitrogen-repressing (PYED medium), or nitrogen-derepressing (proline in the culture medium) conditions (Figs. 1 and 5), indicating that PHS does not affect these two families of major facilitator superfamily of membrane-bound transporters.

Under nitrogen-repressing conditions, PHS strongly inhibited growth of Leu<sup>−</sup> cells and slightly inhibited growth of His<sup>−</sup> cells, indicating that uptake of these two amino acids is diminished by PHS (Fig. 5A). PHS may also inhibit transport of proline by the Put4 protein since growth of prototrophic YPH-A cells was more sensitive to PHS under conditions where proline was the nitrogen source (Fig. 5B) than it was when ammonium ions were the nitrogen source (Fig. 5A). These results, along with the phylogenetic relationship of the amino acid transporters, predict that the activity of most or perhaps all amino acid transporters in *S. cerevisiae* is inhibited by PHS.

Long chain bases have numerous effects on mammalian cells (reviewed in Refs. 4 and 5), and many of these are probably specific for differentiated cells and, therefore, have no counterpart in *S. cerevisiae* cells. Growth inhibition (not cell death) by
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PHS was the end point in our experiments, and this effect has been seen with mammalian cells but the underlying mechanism(s) is unknown (reviewed in Ref. 4). Based upon our data, long chain bases may inhibit mammalian cell growth by blocking uptake of an essential nutrient, perhaps one transported by a membrane-bound protein.

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