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Sphingoid Bases and the Serine Catabolic Enzyme *CHA1* Define a Novel Feedforward/Feedback Mechanism in the Response to Serine Availability*[S]

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Background: The serine deaminase *CHA1* responds to heat stress in a sphingolipid-dependent manner.
Results: *CHA1* requires *de novo* sphingoid base production for induction by serine, limiting growth-suppressing accumulation of sphingoid bases.
Conclusion: Sphingoid bases are feedback sensors of serine availability, forming a feedforward/feedback loop through *CHA1*.
Significance: This study defines a fundamental connection between sphingolipid and amino acid metabolic pathways with implications for disease.

Targets of bioactive sphingolipids in *Saccharomyces cerevisiae* were previously identified using microarray experiments focused on sphingolipid-dependent responses to heat stress. One of these heat-induced genes is the serine deamidase/dehydratase *Cha1* known to be regulated by increased serine availability. This study investigated the hypothesis that sphingolipids may mediate the induction of *Cha1* in response to serine availability. The results showed that inhibition of *de novo* synthesis of sphingolipids, pharmacologically or genetically, prevented the induction of *Cha1* in response to increased serine availability. Additional studies implicated the sphingoid bases phytosphingosine and dihydrosphingosine as the likely mediators of *Cha1* up-regulation. The yeast protein kinases *Pkh1* and *Pkh2*, known sphingoid base effectors, were found to mediate *CHA1* up-regulation via the transcription factor *Cha4*. Because the results disclosed a role for sphingolipids in negative feedback regulation of serine metabolism, we investigated the effects of disrupting this mechanism on sphingolipid levels and on cell growth. Intriguingly, exposure of the *cha1*Δ strain to high serine resulted in hyperaccumulation of endogenous serine and in turn a significant accumulation of sphingoid bases and ceramides. Under these conditions, the *cha1*Δ strain displayed a significant growth defect that was sphingolipid-dependent. Together, this work reveals a feedforward/feedback loop whereby the sphingoid bases serve as sensors of serine availability and mediate up-regulation of *Cha1* in response to serine availability, which in turn regulates sphingolipid levels by limiting serine accumulation.

Sphingolipids constitute a unique class of lipids that have been implicated in a variety of functions in yeast, including nutrient uptake and cell cycle regulation. In particular, several responses to heat stress have been shown to require *de novo* synthesis of sphingolipids, and these include cell cycle arrest, proteolysis, and nutrient import (1–6).

Microarray analysis analyzing the effects of heat stress on gene expression in the *lcb1-100* strain, defective in *de novo* synthesis, was previously used to define genes whose regulation depended on *de novo* synthesis of sphingolipids (7). An especially intriguing gene among these is *CHA1*, which encodes a serine deamidase/dehydratase, known to be up-regulated by exogenous serine (8, 9). This was of great interest because the enzyme serves to attenuate serine levels and because serine also serves as a limiting substrate in the *de novo* synthesis of sphingolipids at 30 °C (6). This mutual sensitivity to serine and the dependence of *CHA1* up-regulation on sphingolipid synthesis suggested the intriguing possibility of a negative feedback mechanism balancing gene expresssion, serine, and sphingolipid levels.

The first step in *de novo* sphingolipid synthesis is the condensation of serine and palmitate by the serine palmitoyl transferase (SPT)[2] complex, followed by reduction, to generate the sphingoid base dihydrosphingosine (DHS), which is then hydroxylated to form the second sphingoid base phytosphingosine (PHS). Ceramide synthases (*Lac1* and *Lag1* in *Saccharomyces cerevisiae*) further modify the sphingoid bases by addition of various fatty acids to generate dihydroceramide and phytoceramide species distinguished by the length, saturation, and/or hydroxylation of the fatty acids in amide linkage. Sphingoid bases are also modified by phosphorylation through the action of sphingoid base kinases (*Lcb4* and *Lcb5* in *S. cerevisiae*) to generate the phosphosphingoid bases. Ceramide species are further modified by addition of the sugars mannose and inositol to form the complex sphingolipids. Combinations of these

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[2] The abbreviations used are: SPT, serine palmitoyl transferase; DHS, dihydrosphingosine; PHS, phytosphingosine; qRT-PCR, quantitative reverse transcription PCR; SC-Thr medium, synthetic complete threonine dropout medium; YPD, yeast extract peptone dextrose.

TABLE 1
Yeast strains used in this work

Strain	Genotype	Source
JK9–3dα	MATα leu2-3,112 ura3-52 rme1 trp1 his4	Laboratory collection
*lcb4*Δ,*lcb5*Δ	MATα leu2-3, 112 ura3-52 rme1 trp1 his4 lcb4::G418 lcb5::G418	Robert Dickson
*cha1*Δ	MATα leu2-3,112 ura3-52 rme1 trp1 his4 cha1::G418	This study
BY4742	MATα his3 leu2 lys2 ura3	Euroscarf
*cha4*Δ	MATα his3 leu2 lys2 ura3 cha4::G418	Euroscarf
Dau15	MATa ura3Δ0 leu2Δ0 trp1Δ his2Δ ade1Δ	Robert Dickson
pkh1-ts,*pkh2*Δ	MATa ura3Δ0 leu2Δ0 trp1Δ his2Δ ade1Δ pkh1^{D398G} pkh2::LEU2	Robert Dickson

covalent modifications produce a vast variety of sphingolipid species, several of which have been implicated in regulation of specific signaling pathways (1). Blocked *CHA1* up-regulation could be due to blocked synthesis of any one of this multitude of lipid species or a subset thereof.

In this study, we aimed to explore the hypothesis that sphingolipid-dependent induction of *CHA1* defines a feedback loop in serine metabolism mediated by sphingolipids. The results show that serine induces *CHA1* through driving *de novo* synthesis of sphingolipids. Moreover, the results show that dysfunction of this pathway leads to hyperaccumulation of intermediate sphingolipids that results in growth suppression in response to high serine. These results support the hypotheses that sphingoid bases serve as sensors of serine availability and that *CHA1* is involved in a novel negative feedback mechanism whereby *Cha1* regulates sphingolipid levels by limiting available serine, and in turn sphingolipids regulate the expression of *CHA1* in response to serine availability.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—Genotypes of wild-type, temperature-sensitive, and deletion mutant strains are described in Table 1. *SUR2* was deleted in JK9-3dα strains to obtain JK9-3dα *sur2*Δ using the following primers (forward, 5′-ACT CCG GGT CTT CGT CTT TAC TG-3′, and reverse, 5′-ACA AAC GTG GGA AGT CGG AGA C-3′) to amplify the *SUR2* locus containing *KanMX* sequence from a genomic DNA of BY4742 *sur2*Δ. For the PCR, the polymerase was activated at 95 °C for 1.5 min, then cycled 35 times with at 55 °C and 72 °C elongation and annealing temperatures, respectively. The PCR product was transformed into JK9-3dα, and then G418-resistant colonies were selected for (yeast lithium acetate transformation methods). The deletion of *SUR2* was verified by PCR and sequencing to confirm the replacement of *SUR2* locus by KanMX sequence. YPD medium was used for the heat stress experiment. For all other experiments, synthetic complete threonine dropout (SC −Thr) medium containing 0.17% yeast nitrogen base (US Biological), 0.5% ammonium sulfate, 2 mM sodium hydroxide, and 0.07% dropout supplement, was used. The dropout supplement contains 5% of the following: adenine, uracil, tryptophan, histidine, arginine, methionine, as well as tyrosine (8%), leucine (15%), lysine (8%), phenylalanine (13%), and aspartate (26%). All dropout components were purchased from Acros Organics (Fisher Scientific), except for methionine and aspartate, purchased from Sigma-Aldrich. Serine powder was added to the medium where specified. PHS and DHS were obtained either from the Medical University of South Carolina (MUSC) Lipidomics Core or Avanti Polar Lipids. Myriocin and serine were purchased from Sigma-Aldrich, fumonisin was purchased from Enzo Clinical Labs (Farmingdale, NY), and aureobasidin was purchased from Clontech. Sphingoid bases were delivered in dimethyl sulfoxide. Myriocin and aureobasidin as in Ref. 10 were delivered in methanol, and fumonisin was delivered in water. For heat stress, cultures were grown in liquid YPD, for serine and sphingoid base induction experiments, cells were grown in liquid SC −Thr (Clontech). For all experiments, cells were grown to mid-log at 30 °C from 1 ml of SC −Thr overnight cultures. Heat stress was performed by shifting cells to a 39 °C water bath. Cultures were harvested by centrifugation at 3500 rpm for 3 min and stored at −80 °C. For spot tests, YPD and SC −Thr plates where prepared with the same composition as for liquid medium, but with 2% agar. Myriocin, aureobasidin, and fumonisin were added at 5, 0.1, and 500 μM, respectively, all plates contain 0.025% methanol vehicle. Plates were spotted from mid-log cultures grown in SC −Thr liquid medium and diluted in water.

RNA and cDNA Preparation—RNA was extracted from 2 × 10^7 cells using the hot acid phenol method (11). Cells were resuspended in 400 μl of TES (Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS) and treated with 400 μl of hot phenol. RNA was further purified using the Qiagen RNeasy RNA cleanup protocol, and the concentration was determined using the $A_{260\ nm}$ after confirming a 260 nm/280 nm absorbance unit ratio of 1.9–2.1. Synthesis of cDNA was carried out with 0.5 μg of total RNA using the Invitrogen SSII first strand cDNA synthesis protocol.

Quantitative Real Time PCR—Real-time PCR was performed in triplicate in 25-μl reactions using the Bio-Rad SYBR Green solution on a Bio-Rad iCycler according to suggested protocols. Cycles were (i) 95 °C for 3 min; (ii) 95 °C for 10 s, 55 °C for 1 min × 40 cycles; (iii) 95 °C for 1 min; and (iv) 55 °C for 1 min. Mean normalized expression was obtained using the Q-gene formulae (12). For serine induction experiments, expression values were normalized relative to the reference gene *RDN18*. Expression for sphingoid base induction experiments were normalized to *ACT1*, due to confounding effects of sphingoid bases on *RDN18*. Primers used are as follows: CHA1 forward, GAT CTC CTC AGG TTT TCG CTA GTT C and reverse, ACC ATT CTC TTC TTT GTC GCT GTA G; RDN18 forward, CCA TGG TTT CAA CGG GTA ACG and reverse, GCC TTC CTT GGA TGT GGT AGC C; ACT1 forward, ATC ACC GCT TTG GCT CCA T and reverse, CCA ATC CAG ACG GAG TAC TTT CTT.

Measurement of Sphingoid Bases and Ceramides—Cells were grown to mid-log, treated, and harvested at 3500 rpm for 3 min, and then pellets were flash frozen in a methanol/dry ice bath. 1.19 × 10^9 cells were resuspended in 1 ml of the extraction solvent (ethanol, diethyl ether, pyridine, ammonium hydrox-

ide, water (50:10:2:25:15)), placed in screw-cap vials containing 0.5 ml of glass beads (Sigma), and vortexed at 4 °C at maximum speed for 12 min to homogenize. The resulting suspension was diluted by adding an additional 1 ml of extraction solvent, incubated at 60 °C for 15 min, and centrifuged at 3500 rpm for 10 min at room temperature, and then the supernatant was transferred to a fresh tube. The pellet was resuspended in a fresh 2 ml of extraction solvent and then incubated and centrifuged as above. The two supernatants were combined, and the extract was dried down. Lipids were quantified according to the method described in Ref. 13. Lipid levels were normalized to total protein.

Measurement of Total Protein—Culture aliquots containing 1.19×10^7 cells were harvested in 1.5-ml screw-cap vials. Cells were pelleted and resuspended in 500 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM DTT, 1 mM PMSF, and Protease Inhibitor Mixture), 250 μl of glass beads were added, and cells were homogenized by vortexing at 4 °C for 12 min total, in 3-min intervals. Lysate was centrifuged at 600 × *g* for 10 min, and total protein in the supernatant was measured by Bio-Rad BCA protein assay using BSA as a calibration standard.

Analysis of Complex Sphingolipids—Cells were grown to mid-log in SC −Thr medium and treated with medium containing 10 or 50 mM serine and 20 μCi/ml [^{3}H]inositol (American Radiochemicals). 6.5×10^7 cells were spun and extracted by the Mandala method (14), then resuspended in 30 μl of 2:1 chloroform and methanol, and the total volume was spotted on a TLC plate (Whatman 60 Å, 250 μm, 20-cm LK6D). Lipids were separated in (9:7:2) chloroform, methanol, and ammonium hydroxide (4.2 N). Plates were developed on x-ray film (Thermo Scientific) at −80 °C for 48 h. Spots were identified by comparing samples labeled with [^{3}H]inositol *versus* those labeled with [^{3}H]serine (supplemental Fig. S3) and by comparison with published data (15–17). Lipids were quantified by densitometry using ImageJ software. Spot density was converted to moles of lipid by calibrating spot densities containing a known quantity of [^{3}H]inositol.

Measurement of Intracellular Serine—Cells were grown to early log in SC −Thr, transferred to fresh medium containing 0, 10, or 50 mM serine, grown for 21 h, and then 2.6×10^8 cells were harvested by centrifugation at 3500 × *g* for 3 min. Cells were quenched by methanol chilled to −40 °C, spun for 30 s at 16,100 × *g*, then washed again with cold methanol to remove residual serine left from the medium (18). Serine was extracted by resuspending and boiling in 1 ml of deionized water according to Ref. 19. Intracellular serine was measured by the method described in Ref. 20 employing a Waters 717plus/1525/2487 HPLC autosampler/pump/detector, with some modifications: amino acids were separated on an ES Industries Chromega 150 × 4.6- mm, MC18, 60-Å column. Serine was identified and quantified using the Amino Acid H standard mixture (Pierce) as a calibration standard.

Statistical Analysis—AVOVA with the Bonferroni post test was used to assign significance at $p < 0.01$ or $p < 0.05$ as specified. The specific statistical method and the number of replicates for each experiment are indicated in the figure legends.

RESULTS

De Novo Sphingolipid Synthesis Mediates Induction of CHA1 in Response to Serine—In a genomic approach, it was shown previously that heat stress up-regulates the serine deamidase/dehydratase *CHA1* but that this up-regulation fails in the *lcb1-100* mutant, which has a defective SPT complex and thus cannot efficiently carry out the first step of *de novo* sphingolipid synthesis (7). However, the *lcb1-100* mutant also exhibits baseline effects on sphingolipids, and therefore, it does not fully discriminate acute effects of sphingolipids. Therefore, to evaluate the role of acute *de novo* synthesis directly, we employed myriocin, a pharmacological inhibitor of SPT. Yeast cells were subjected to heat stress in the presence or absence of myriocin, and the levels of *CHA1* RNA were quantitated using quantitative RT-PCR (qRT-PCR). The results showed that myriocin significantly blocked the up-regulation of *CHA1* in response to heat stress (Fig. 1*A*). Taken together, these results establish that acute *de novo* synthesis is required for heat induction of *CHA1*.

CHA1 is known to be strongly up-regulated by serine (8, 21), and we have shown previously that effects of heat stress on sphingolipids are specifically mediated by heat-induced increases in serine uptake (6). Thus, it became important to determine whether *CHA1* induction by serine in the absence of heat stress requires its incorporation into sphingolipids, as this would imply participation of sphingolipids in a negative feedback mechanism regulating serine metabolism. To address this hypothesis, cells were treated with serine at several doses of myriocin, and *CHA1* expression was measured by qRT-PCR. As shown in Fig. 1*B*, myriocin blocked C*HA1* up-regulation by serine in a dose-dependent manner with 50% inhibition observed at a concentration of 0.5 μM myriocin, indicating that sphingolipid synthesis is required for *CHA1* up-regulation by serine in the absence of heat. To further confirm that myriocin blocks *CHA1* up-regulation specifically by blocking the SPT activity, the temperature-sensitive *lcb1-100* strain, which has defective SPT activity, and its wild-type background strain RH406 were treated with serine, and *CHA1* expression was measured at semipermissive temperature. *CHA1* was up-regulated dramatically in the background strain RH406 but completely blocked in the *lcb1-100* mutant (Fig. 1*C*). The semipermissive temperature of 33 °C is sufficient to block *lcb1-100* activity without stimulating the heat stress response. For all experiments where serine was used to induce *CHA1*, cells were grown and treated in SC −Thr medium to avoid high background induction of *CHA1* by threonine, which also induces this gene. The results showing that *CHA1* up-regulation requires *de novo* sphingolipid synthesis in the absence of heat stress demonstrate that sphingolipids are the key mediators of the induction of *CHA1* by serine.

Sphingoid Bases PHS and DHS Mediate CHA1 Up-regulation—To determine which class of sphingolipids is required for *CHA1* up-regulation by serine, the effects of serine on *CHA1* were determined in several mutants deleted in key enzymes of the sphingolipid metabolic pathway. Specifically *CHA1* was induced by serine in the *tsc3*Δ, *lcb4*Δ,*lcb5*Δ, *lag1*Δ, *lac1*Δ, and *sur2*Δ strains, and expression was measured by qRT-PCR (Fig. 2*A*). The first strain tested, *tsc3*Δ, is deleted in a nonessential

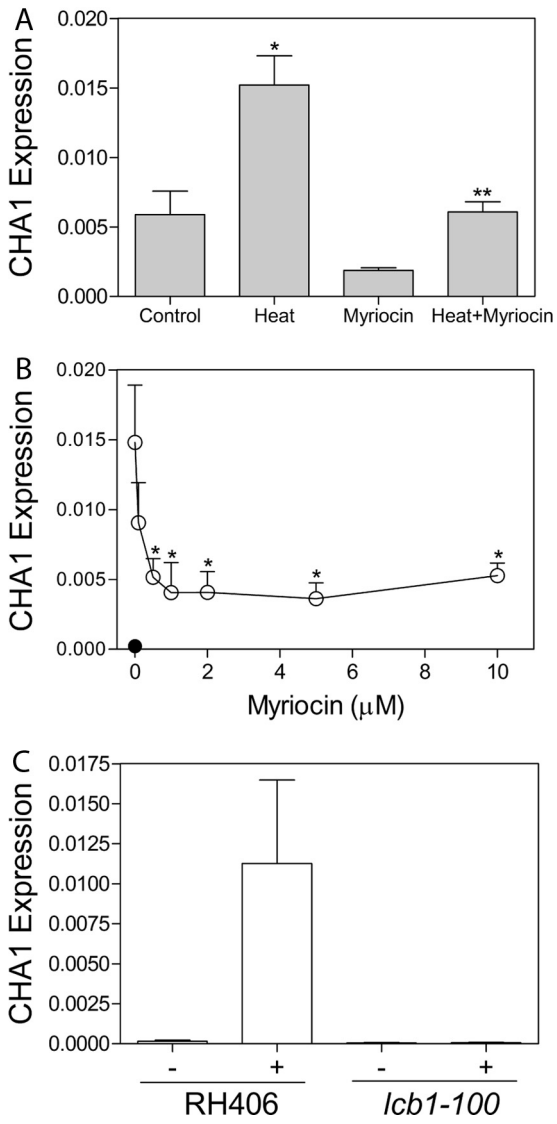

FIGURE 1. ***De novo* sphingolipid synthesis is required to mediate *CHA1* induction in response to heat stress and to serine availability.** *A*, *CHA1* expression was measured by qRT-PCR before and after induction by heat stress with and without myriocin. Cells were grown to mid-log in YPD, pretreated with 5 μM myriocin (or vehicle) for 45 min, and then shifted from 30 to 39 °C for 15 min. The difference in expression between control and heat stress is significant (*), and the difference in expression between heat and myriocin plus heat is significant (**) by one-way ANOVA, $p < 0.05$. *B*, *CHA1* was induced by serine over a range of myriocin doses from 0 to 10 μM (*open circles*); expression without induction by serine is shown (*filled circle*). JK9-3dα cells were grown to mid-log phase in SC −Thr, pretreated for 45 min with the specified dose of myriocin or vehicle, and then induced with 0.5 mM serine for 15 min. * Indicates that the difference between the vehicle (zero) and the indicated data point is significant as determined by one-way ANOVA, $p < 0.05$. *C*, *lcb1-100* mutant and RH406 background strain were treated with serine to determine the degree of *CHA1* induction. Cells were grown to mid-log in SC −Thr medium and then induced with 0.5 mM serine for 15 min. The difference between the wild-type and mutant expression after treatment was confirmed to be significant by one-way ANOVA at $p < 0.01$. For all experiments data presented are the average ± S.E. (*error bars*) of at least three independent experiments.

subunit of the SPT complex, which has diminished SPT activity. The *Tsc3* subunit is also implicated in formation of the less abundant C20 sphingoid bases (6), which have a 20-carbon chain. The *tsc3*Δ strain did not show up-regulation significantly different from wild type, disfavoring the C20 sphingoid bases as possible mediators. The *lcb4*Δ,*lcb5*Δ double deletion strain, deleted in both sphingoid base kinases *LCB4* and *LCB5*, showed up-regulation that was not significantly different from wild type, implying that the sphingoid base phosphates are not required for mediating *CHA1* expression. This is a relevant conclusion because previous work has implicated PHS-1-phosphate in up-regulation of specific genes during heat stress (4, 22, 23) and therefore distinguishes DHS/PHS *versus* PHS-1-phosphate-regulated pathways. The *lag1*Δ and *lac1*Δ strains deleted in the ceramide synthases showed normal up-regulation, disfavoring the possibility that ceramide synthesis is required for *CHA1* up-regulation. It also argues against the possibility that complex sphingolipid production is necessary to mediate induction. Moreover, the *sur2*Δ strain deleted in the sphingoid base/ceramide hydroxylase that hydroxylates DHS and dihydroceramide into PHS and phytoceramide also showed complete up-regulation. Because the result on *lag1*Δ and *lac1*Δ disfavors ceramide, the *sur2*Δ result implies that either PHS or DHS could potentially mediate *CHA1* up-regulation. Up-regulation of *CHA1* in the other mutant strains tested implies that production of the sphingoid bases DHS and/or PHS is the specific reaction required for complete *CHA1* induction.

To confirm the conclusion derived from analysis of mutant strains, the pharmacological inhibitors fumonisin (Fig. 2*B*) and aureobasidin (Fig. 2*C*), inhibitors of ceramide (24) and complex sphingolipid synthesis (25), were employed, respectively. Neither inhibitor blocked *CHA1* up-regulation (in contrast to myriocin). A small loss of up-regulation was observed at 1 mM fumonisin, but this is in excess of concentrations required to inhibit ceramide synthesis efficiently in *S. cerevisiae* (22, 26). Negative inhibition of up-regulation by fumonisin further consolidates the results with the *lag1*Δ and *lac1*Δ single mutants which still harbor some ceramide synthase activity; however, the *lag1*Δ,*lac1*Δ double mutant is very difficult to produce, and the only such strain available proved problematic, therefore demonstrating the effect of acute inhibition is critical to eliminating ceramide synthesis as a critical reaction for *CHA1* up-regulation. Lack of inhibition of up-regulation of *CHA1* by aureobasidin or by fumonisin provides strong evidence that formation of complex sphingolipids is not necessary for *CHA1* up-regulation.

The above results strongly suggest that the sphingoid bases are necessary for *CHA1* up-regulation. Therefore, it became important to determine whether DHS/PHS are also sufficient for up-regulation. To address this issue, the effects of the exogenous sphingoid bases DHS and PHS on *CHA1* expression were evaluated by measuring dose- and time-dependent *CHA1* induction by PHS/DHS. *CHA1* was transiently up-regulated by both sphingoid bases, but the change in expression was variable (supplemental Fig. S1).

Yeast Protein Kinases Pkh1 and Pkh2 Mediate CHA1 Up-regulation via Transcription Factor Cha4—Previous work has implicated the yeast protein kinases *Pkh1* and *Pkh2* in mediating sphingoid base-dependent gene regulation (27–29), making them likely candidates as mediators of *CHA1* up-regulation. *Pkh1* and *Pkh2* form a redundant but essential pair. A strain deleted in *PKH2*, but carrying a temperature-sensitive *pkh1-ts* allele was used to test the effects of loss of kinase function.

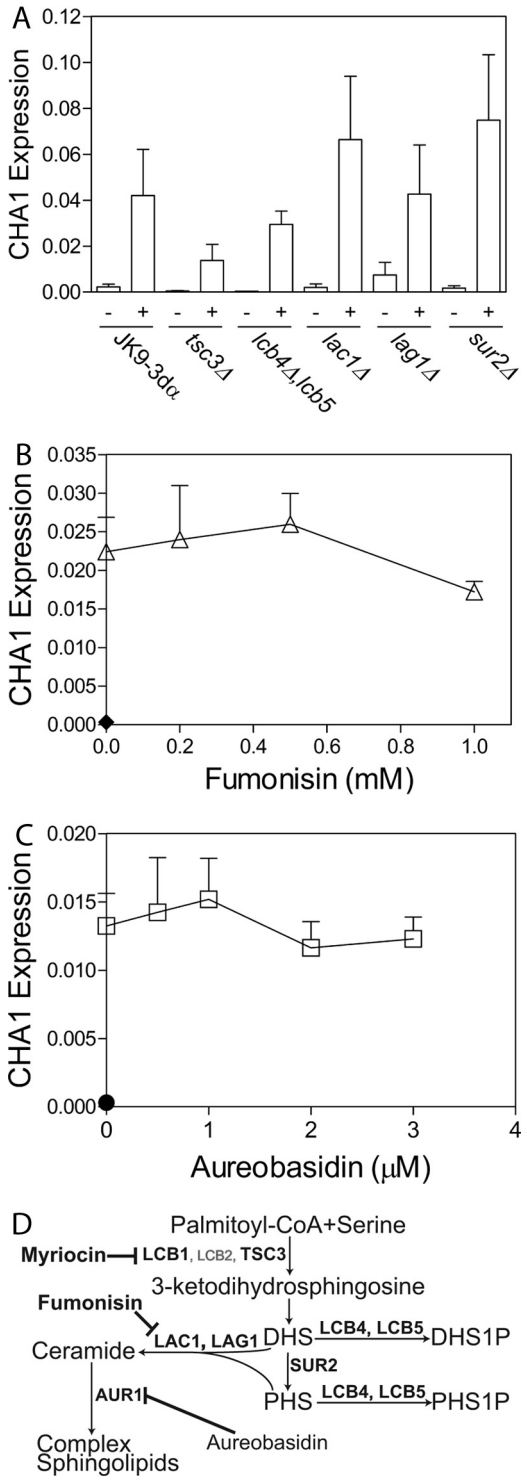

FIGURE 2. **Sphingoid bases and not ceramide or phosphosphingoid bases are required for *CHA1* up-regulation.** *CHA1* was induced by serine in key mutant strains deleted in key sphingolipid enzymes and after treatment with pharmacological inhibitors in the JK9-3dα background. *A*, wild-type and mutant strains were grown to mid-log in SC −Thr and induced with 0.5 mM serine for 15 min, and expression was measured by qRT-PCR. *B* and *C*, for fumonisin and aureobasidin treatment, cells were pretreated for 2 h with drug or vehicle (0.02% methanol) before induction. *B*, cells were not induced (*filled diamond*) or induced with serine after treatment with 0, 0.2, 0.5, and 1.0 mM fumonisin (*open triangles*), or 0, 0.5, 1, 2, and 3 μM aureobasidin (*C*) (*open squares*); expression with no induction is shown (*filled circle*). Induction by serine was found to be significant at $p < 0.01$ by one-way ANOVA in all samples. *CHA1* expression was not significantly different in any of the mutant strains after induction at $p < 0.05$ confidence determined by two-way ANOVA. Neither pharmacological inhibitor showed significant inhibition of induction at $p < 0.05$ confidence by one-way ANOVA. Data presented are the average ± S.E. (*error bars*) of at least triplicate experiments. *D*, sphingolipid metabolic pathway illustrates the key points where the pathway was disrupted in this study using inhibitors or mutant strains indicated in *bold font*.

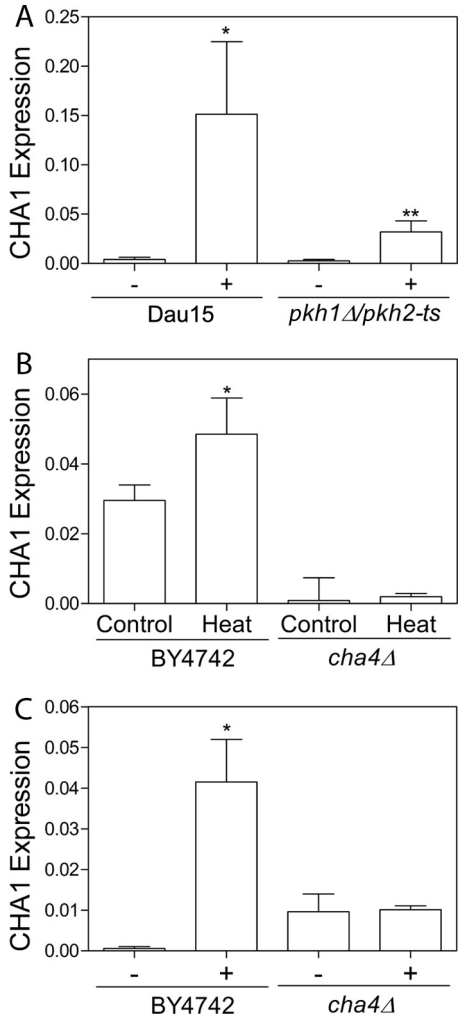

FIGURE 3. **Increased sphingoid base signals *CHA1* induction by transcription factor *Cha4* via kinases *Pkh1* and *Phk2*.** *CHA1* expression was measured by qRT-PCR after a 15-min induction with 0.5 mM serine in the *pkh1-ts,pkh2*Δ strain, in the *cha4*Δ strain and in their respective background strains Dau15 and BY4742. *A*, *pkh1-ts,pkh2*Δ strain and its background strain were shifted to 37 °C during the 15-min induction with serine. *B* and *C*, *cha4*Δ and its background strain were induced with 0.5 mM serine for 15 min at 30 °C in SC −Thr (*C*) or by 15 min at 39 °C in YPD (*B*). * Indicates that CHA1 was significantly up-regulated by serine or heat treatment in each background strain. ** Indicates that the difference in expression between Dau15 and *pkh1-ts,pkh2*Δ after treatment is significant. In both cases significance was determined by one-way ANOVA, $p < 0.05$. Data presented are the average ± S.E. (*error bars*) of triplicate experiments.

CHA1 up-regulation in response to serine was markedly reduced in the *pkh1-ts,pkh2*Δ mutant strain, implying that the PKH kinases play a critical role in the up-regulation of *CHA1* (Fig. 3*A*).

The transcription factor *Cha4* is known to regulate *CHA1* up-regulation (30, 31). Therefore, it was of interest to determine whether *Cha4* is the key transcription factor mediating induction by heat as well as serine. *CHA1* up-regulation by serine was measured in the *cha4*Δ deletion strain and in its BY4742

TABLE 2

Sphingoid bases and ceramides accumulate in *cha1*Δ with high serine load

The *cha1*Δ and JK9–3dα strains were grown in SC −Thr medium containing no serine or supplemented with 10 or 50 mM serine for 21 h. Equal numbers of cells for each treatment were harvested, extracted, quantified by HPLC-tandem MS, and normalized to total protein. Before treatment, cells were diluted from overnight cultures and grown to early log phase. Bold type indicates where the difference between *cha1*Δ and wild-type values is significant at $p < 0.05$ by one-way ANOVA. Data presented are the average ± S.E. of at least triplicate experiments.

Metabolite	−Thr D.O. media		10 mM serine		50 mM serine	
	JK9-3dα	*cha1*Δ	JK9-3dα	*cha1*Δ	JK9-3dα	*cha1*Δ
	pmol/μg protein	*pmol/μg protein*	*pmol/μg protein*	*pmol/μg protein*	*pmol/μg protein*	*pmol/μg protein*
Phytoceramide	10.5 ± 0.6	12.2 ± 0.3	9.1 ± 0.5	6.1 ± 0.2	8.95 ± 0.27	**30.0 ± 1.6**
Dihydroceramide	42.1 ± 0.9	41.1 ± 2.0	29.5 ± 1.7	27.9 ± 2.2	18.3 ± 1.1	**45.2 ± 2.4**
Phytosphingosine	3.35 ± 0.44	3.02 ± 0.4	5.6 ± 1.0	6.6 ± 1.0	3.63 ± 0.9	**12.0 ± 3.1**
Dihydrosphingosine	12.7 ± 0.75	13.0 ± 1.3	6.9 ± 0.7	6.9 ± 0.7	5.02 ± 1.2	**21.5 ± 5.3**
Serine ($\times 10^{-3}$)	0.60 ± 0.01	0.57 ± 0.01	0.92 ± 0.02	1.43 ± 0.19	6.71 ± 1.09	**13.1 ± 1.7**

background strain, and it was found that up-regulation was blocked in *cha4*Δ, confirming its role in transcriptional activation (Fig. 3*B*). *CHA1* was also induced in BY4742 by shifting to 39 °C for 15 min in YPD, and this induction was dependent on *Cha4* (Fig. 3*C*). Thus, these results show that *Cha4* is a key transcription factor in the induction of *CHA1* in response to both heat stress and serine availability.

*Serine Hyperaccumulation Leads to Hyperaccumulation of Sphingoid Bases and Ceramide in cha1*Δ *Strain under High Serine Load*—The demonstration that serine is important for sphingolipid regulation coupled with the fact that *Cha1* acts on serine led us to the hypothesis that induction of *CHA1* serves not only to attenuate serine levels (thus defining a feedback pathway in serine metabolism), but also that *CHA1* may then indirectly feed back on sphingolipid levels (see Fig. 6 for scheme). This speculation raises the more direct question of how the presence or absence of *Cha1* might affect sphingolipid levels in the presence of high serine. To address this question, *cha1*Δ and its background strain JK9-3dα were grown in media with either a zero, low (10), or high (50 mM) serine concentration. The low serine condition corresponds to a concentration of serine typically found in rich media. Sphingolipids were extracted and measured by HPLC-tandem MS, and serine was extracted and measured by HPLC. It was found that after 21 h of growth, the mutant strain had accumulated 2–4-fold higher levels of both DHS, PHS as well as dihydroceramide and phytoceramide (Table 2), and 2-fold higher levels of serine. These results demonstrate that sphingolipid levels are sensitive to intracellular serine and that *Cha1* is vital to regulating sphingolipid levels by way of regulating intracellular serine levels.

Next, the effects of *Cha1* on regulating complex sphingolipid levels were evaluated using tritiated inositol to label the inositol-containing complex sphingolipids. The identified and labeled spots are phosphatidylinositol, inositol phosphoceramide, mannose-(inositol phospho)-ceramide (MIPC), and mannose-(inositol phospho)$_2$-ceramide, and the results of three replicate experiments are shown (Fig. 4). No significant difference was observed between WT and *cha1*Δ (Table 3).

*Cha1*Δ *Strain Shows Sphingolipid-dependent Sensitivity to High Serine*—Based on the above results, we predicted that feedback regulation of sphingolipid synthesis by *CHA1* exerts a physiologic function in attenuating possible effects of sphingoid bases on growth, especially because these molecules have been implicated in regulation of cell cycle and growth in yeast (3, 32, 33). To test this prediction, a spot test was used to demonstrate sensitivity of the *cha1*Δ strain to high serine load. Relative to the WT, the *cha1*Δ strain showed almost no growth on high serine (50 mM) plates, but was able to grow normally on YPD or low serine (10 mM) plates (Fig. 5). To determine whether this sensitivity to high serine was due to accumulation of excess sphingolipids, plates were also prepared with 5 μM myriocin. The presence of myriocin almost completely rescued the serine sensitivity phenotype (Fig. 5), implying that in the absence of *Cha1*, high serine leads to cell death arising from unregulated sphingolipid production.

Although the results on *CHA1* induction implicated sphingoid bases as the active species, it is not necessary that they are also the species involved in the growth suppression, which may also be mediated by the observed increase in ceramides or other sphingolipids. Therefore, in addition to myriocin, fumonisin

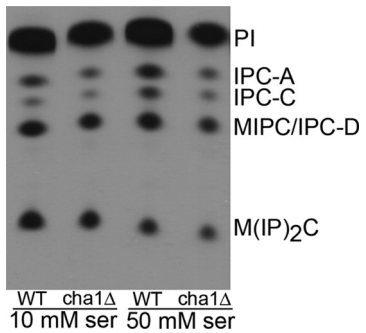

FIGURE 4. **Complex sphingolipids do not accumulate in *cha1*Δ with high serine.** The *cha1*Δ and JK9-3dα (WT) strains were grown in high and low serine media with 20 μCi of [^{3}H]inositol for 21 h. Equal cells were extracted, separated by TLC, and visualized on x-ray film. The film shown is representative of triplicate experiments. *PI*, phosphatidylinositol; *IPC*, inositol phosphoceramide; *MIPC*, mannose-(inositol phosphoceramide); *M(IP)$_2$C*, mannose-(inositol phospho)$_2$-ceramide.

TABLE 3

High serine availability does not lead to accumulation of complex sphingolipids

The *cha1*Δ and JK9-3dα strains were grown in SC −Thr medium containing 10 or 50 mM serine and 20 μCi of [^{3}H]inositol for 21 h. Equal cells were extracted, separated by TLC, visualized on x-ray film, and quantified by densitometry. Data presented are the average ± S.E. of triplicate experiments.

Lipid/10^6 cells[a]	10 mM serine		50 mM serine	
	JK9-3dα	*cha1*Δ	JK9-3dα	*cha1*Δ
	pmol	*pmol*	*pmol*	*pmol*
IPC-A	0.07 ± 0.03	0.09 ± 0.00	0.07 ± 0.03	0.09 ± 0.01
IPC-C	0.09 ± 0.02	0.12 ± 0.60	0.06 ± 0.02	0.08 ± 0.03
MIPC/IPC-D	0.06 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.00
M(IP)$_2$C	0.13 ± 0.01	0.11 ± 0.07	0.08 ± 0.06	0.08 ± 0.07

[a] IPC, inositol phosphoceramide; MIPC, mannose-(inositol phosphoceramide); M(IP)$_2$C, mannose-(inositol phospho)$_2$-ceramide.

and aureobasidin were used to determine whether synthesis of ceramide and complex sphingolipoids, respectively, are required to give rise to the serine sensitivity phenotype. However, neither fumonisin nor aureobasidin showed rescue of the phenotype to a degree similar to that of myriocin (Fig. 5) at concentrations where inhibition of lipid synthesis was confirmed (supplemental Fig. S4). Only inhibition of *de novo* sphingolipid synthesis at the initial step led to rescue of the high serine phenotype, thus implying that accumulation of the sphingoid bases specifically leads to the serine sensitivity phenotype.

DISCUSSION

This study reveals that sphingolipid synthesis is necessary for mediating the induction of the serine deamidase/dehydratase *Cha1* in response to increased serine availability and/or uptake, which can arise from increased extracellular serine or in response to heat stress (6), respectively. The results implicate the sphingoid bases PHS/DHS as the likely key sphingolipid species required for induction illustrated in the *left panel* of Fig. 6. Moreover, the results show that the *cha1*Δ strain is unable to regulate sphingolipid synthesis, leading to hyperaccumulation of sphingoid bases and ceramides when serine availability is high. This unregulated sphingolipid synthesis leads to growth inhibition under the same conditions, as illustrated in the *right panel* of Fig. 6.

These results indicate the presence of a feedforward/feedback mechanism tying together the regulation of serine and sphingolipid metabolism. In turn, the results carry implications for human disease, including cancer, central nervous system (CNS) development, and neurological disease.

A major conclusion from this study is that sphingoid bases function as key sensors and signaling mediators of serine availability. It was previously shown that *CHA1* is up-regulated by increased availability of serine (8), which was confirmed in this work. The results from this study demonstrate, by blocking *de novo* sphingolipid synthesis using myriocin and the *lcb1-100* mutant, that sphingolipid synthesis is necessary for induction of *CHA1* in response to increased serine availability. Moreover, previous results from our group showed that exogenous serine drives *de novo* synthesis of sphingolipids (6). Taken together, these results show that endogenous sphingolipids respond directly to exogenous serine levels and then mediate the effects of serine on *CHA1* induction; thus fulfilling criteria as "sensors" of serine availability.

Interestingly, previous work has shown that sphingolipid synthesis is up-regulated by heat stress (23), and previous work from our group has shown that this effect requires a heat-mediated increase in serine uptake (6, 22). Moreover, *CHA1* was one of the genes that were highly induced in response to acute heat stress (7). Therefore, this proposed role of sphingolipids in sensing serine extends to heat stress and possibly other mechanisms that may regulate serine availability. Such mechanisms now appear to employ this intrinsic mechanism of coupling serine to sphingolipids and then to downstream targets.

The sphingoid bases are the most likely mediators of *CHA1* induction based on the genetic and pharmacological data presented here. Specifically, the inhibitor myriocin as well as the *lcb1-100* mutant, both of which act upstream to block sphingoid base production, block or partially block *CHA1* induction.

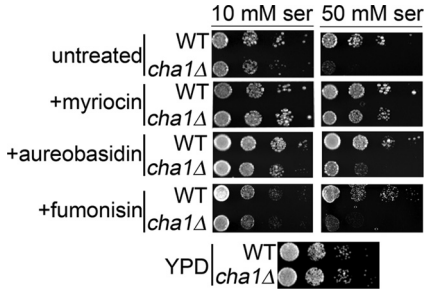

FIGURE 5. ***cha1*Δ strain shows a sphingoid base-dependent sensitivity to high serine.** The *cha1*Δ and JK9-3dα (WT) strains were spotted on agar plates made with SC −Thr medium supplemented with 10 and 50 mM serine. Matched plates were made with myriocin, aureobasidin, fumonisin, or vehicle. An additional YPD plate shows growth in the absence of nutrient limitation or drug treatment. Plates were incubated at 30 °C for 3 days. Cells were grown in liquid SC −Thr medium to mid-log phase and diluted in water to an A_{600} of 0.3, and then 5 μl of successive 10:1 dilutions were spotted with the most to least dilute cell suspensions arranged from *left* to *right*. Plates shown are representative of five independent experiments.

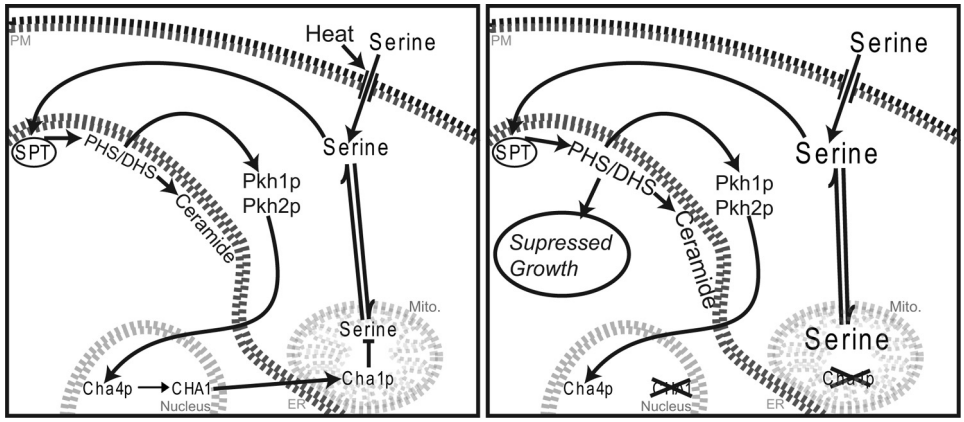

FIGURE 6. **Feedforward/feedback model for co-regulation of sphingolipid synthesis and *CHA1* expression.** *Left*, feedforward/feedback model maintains serine and sphingolipid homeostasis in the wild-type cell. Serine is taken up through the plasma membrane (*PM*), stimulated by heat stress or by increased serine in the medium. Intracellular serine passes into the endoplasmic reticulum (*ER*) and interacts with the SPT complex. The resulting increase in sphingoid bases stimulates increased *CHA1* expression via *Pkh1/Pkh2* and *Cha4*. *Cha1* increases in the mitochondria (*Mito.*) and catabolizes serine, affecting total intracellular serine. *Right*, homeostasis breaks down in the *cha1*Δ strain under high serine load. Loss of *Cha1* results in serine hyperaccumulation leading to hyperaccumulation of the sphingoid bases and ceramide and leading to suppressed growth.

Other mutants and inhibitors, which block lipid production downstream of the sphingoid bases, have no effect (Fig. 2, *A–C*). Thus, this combined genetic and pharmacologic approach "isolates" sphingoid bases as the likely mediators. Moreover, PHS/DHS were sufficient for *CHA1* induction. Thus, sphingoid bases are necessary and sufficient for induction of *Cha1* in response to increased serine availability.

Mechanistically, the results implicate the kinases *Pkh1* and *Pkh2* as mediators of *CHA1* induction downstream of the sphingoid bases. Activation of *Pkh1/Pkh2* by the sphingoid bases has been implicated in several cell functions, including protein synthesis upon recovery from heat stress (34), P-body formation (35, 36), cell wall integrity, and endocytosis (37, 38). Other studies have implicated a specific phosphorylation site on *Ypk1*, the primary substrate of *Pkh1*, in mediating sphingolipid-mediated signaling (39), and PHS was shown to activate *Pkh1 in vitro* (32); however, some doubt has been raised regarding the latter (40).

Because *Cha1* acts to decrease the levels of serine, the results on induction of *Cha1* by serine through sphingolipids suggested to us that dysfunction of this homeostatic mechanism would result not only in hyperaccumulation of serine but also in derangements in sphingolipid metabolism. Indeed, serine levels were elevated in the absence of *CHA1* expression in response to increased extracellular serine (Table 2). Importantly, the levels of sphingoid bases and ceramides were also significantly elevated under the same conditions, providing further evidence for the coupling of serine metabolism and sphingolipid metabolism. Complex sphingolipids in contrast did not increase under the same conditions, consistent with a recent study (41) where hyperaccumulation of ceramides and sphingoid bases was not accompanied by accumulation of complex sphingolipids. Functionally, the loss of feedback control of serine and sphingolipids in the *CHA1* mutant led to a growth defect on high serine media. This growth defect, due to hyperaccumulated intracellular serine, was mediated by sphingolipids and most likely sphingoid bases. Thus, the results reveal a fundamental role for sphingolipids, as direct downstream metabolites of serine, in sensing serine availability, and then initiating a feedback mechanism to induce *Cha1*. CHA1 expression then serves to attenuate serine levels and consequently sphingolipid levels. It should be noted that the action of *CHA1* also provides a nitrogen source (8) and diverts serine toward other metabolites (via conversion to pyruvate) (9, 21). It is intriguing to speculate that sphingolipids may have primarily evolved to sense (metabolically) and regulate (via signaling) these fundamental metabolic feedback pathways.

The close relationship between serine and sphingolipid levels presented here suggests a fundamental connection between amino acid and sphingolipid metabolic pathways. Preliminary data from our laboratory, which led us to focus on *CHA1*, also identified a number of other genes involved in many aspects of amino acid metabolism/catabolism that are regulated in a sphingolipid-dependent manner. PHS in yeast and ceramide in mammalian cells have been previously shown to regulate amino acid uptake by regulating levels of amino acid transporters (2, 42). The homeostatic mechanism described here may therefore affect amino acid transporter activity and vice versa. Together, these data suggest a complex interwoven connection between amino acid and sphingolipid metabolic pathways.

The feedback mechanism revealed in this study may also have implications for cancer, CNS development, and neurological disease (43). The human *CHA1* homolog hSDH is expressed in lung cancer cells as cSDH, a mutant enzyme with lower activity (44, 45). The effect of the cSDH mutation on intracellular serine has not been studied; however, defective serine metabolism has been associated with a number of cancers (46–48). The cSDH mutant enzyme also has altered amino acid substrate specificity, favoring threonine over serine (44, 45). Altered amino acid specificity of a mutant form of the human SPT subunits, favoring glycine and alanine over serine, leads to formation of deoxysphinganine and de-(oxymethyl)-sphinganine, leading to hereditary sensory type I neuropathy (49, 50). Because altered availability of glycine and alanine leads to production of deoxysphinganine and de-(oxymethyl)sphinganine, disruption of their metabolism may also potentially lead to neurological disease.

In conclusion, this study reveals the existence of a novel regulatory mechanism whereby sphingoid bases indirectly regulate serine levels by controlling *CHA1* expression, and *Cha1* expression in turn indirectly regulates sphingolipid levels by regulating serine levels. Importantly, these mechanisms rely on the direct coupling of serine to sphingolipid metabolism. It was also concluded that this homeostatic mechanism is essential for cell growth regulation under high serine load.

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