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Modulation of Sphingolipid Metabolism by the Phosphatidylinositol-4-phosphate Phosphatase Sac1p through Regulation of Phosphatidylinositol in *Saccharomyces cerevisiae*[*] [S]

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Sphingolipids and phosphoinositides both play signaling roles in *Saccharomyces cerevisiae*. Although previous data indicate independent functions for these two classes of lipids, recent genetic studies have suggested interactions between phosphatidylinositol (PtdIns) phosphate effectors and sphingolipid biosynthetic enzymes. The present study was undertaken to further define the effects of phosphatidylinositol 4-phosphate (PtdIns(4)P) metabolism on cell sphingolipid metabolism. The data presented indicate that deletion of SAC1, a gene encoding a PtdIns(4)P phosphatase, increased levels of most sphingolipid species, including sphingoid bases, sphingoid base phosphates, and phytoceramide. In contrast, *sac1*Δ dramatically reduced inositol phosphosphingolipids, which result from the addition of a PtdIns-derived phosphoinositol head group to ceramides through Aur1p. Deletion of SAC1 decreased PtdIns dramatically in both steady-state and pulse labeling studies, suggesting that the observed effects on sphingolipids may result from modulation of the availability of PtdIns as a substrate for Aur1p. Supporting this hypothesis, acute attenuation of PtdIns(4)P production through Stt4p immediately increased PtdIns and subsequently reduced sphingoid bases. This reduction was overcome by the inhibition of Aur1p. Moreover, modulation of sphingoid bases through perturbation of PtdIns(4)P metabolism initiated sphingolipid-dependent biological effects, supporting the biological relevance for this route of regulating sphingolipids. These findings suggest that, in addition to potential signaling effects of PtdInsP effectors on sphingolipid metabolism, PtdIns kinases may exert substantial effects on cell sphingolipid profiles at a metabolic level through modulation of PtdIns available as a substrate for complex sphingolipid synthesis.

Sphingolipids play vital roles in *Saccharomyces cerevisiae*, including regulation of translation, cell cycle, sporulation, ubiquitin-dependent proteolysis, actin cytoskeleton rearrangements, endocytosis, stress responses, and numerous other processes (see Refs. 1–3 for review). Phosphoinositides comprise another group of key lipid mediators that regulate numerous cellular functions, including organization of the actin cytoskeleton, endocytosis, cytokinesis, vacuolar morphology, and translation initiation (4–7). The apparent overlap in biological roles of these distinct classes of lipids implies interaction between them. Indeed, recent studies support regulation of sphingolipid metabolism by phosphoinositides (8–11).

Sphingolipid metabolism has been reviewed extensively, but, in brief, sphingolipid synthesis begins with the condensation of palmitoyl-coenzyme A and serine by serine-palmitoyl transferase to form 3-ketodihydrosphingosine (12) (Fig. 1). This short-lived intermediate is quickly converted to the sphingoid bases dihydrosphingosine (DHS)[2] and phytosphingosine (PHS), which undergo phosphorylation to form sphingoid base phosphates or *N*-acylation to form ceramides (13, 14). Phytoceramide and phosphatidylinositol (PtdIns) are used as substrates by Aur1p to form inositol phosphorylceramide (IPC), which may then be converted into other complex sphingolipids (15).

PtdIns and its derivatives serve well established signaling roles in both mammalian cells and *S. cerevisiae*, which contains three PtdIns 4-kinases producing distinct pools of PtdIns(4)P (5): Stt4p, which resides at the plasma membrane (16); Pik1p, which is primarily located at the Golgi (17); and Lsb6p, which is located at the plasma and vacuolar membranes (18). Notably, Stt4p is the only wortmannin-sensitive PtdIns 4-kinase in *S. cerevisiae* (19). Additionally, the PtdIns(4)P 5-kinase Mss4p uses PtdIns(4)P produced by Stt4p as its substrate (5). Importantly, the Sac1p lipid phosphatase, which localizes to the endoplasmic reticulum, primarily regulates the pool of PtdIns(4)P generated by Stt4p *in vivo* (20) (Fig. 1).

Some interactions between PtdIns pathways and sphingolipid metabolism have been probed using yeast genetic strategies, and several recent studies have focused on regulation of sphingolipid metabolic processes by PtdIns(4,5)P_2 effector signaling (see Ref. 21 for review). PtdIns(4,5)P_2 effectors have been

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: DHS, dihydrosphingosine; IPC, inositol phosphorylceramide; PHS, phytosphingosine; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P, phosphatidylinositol 4,5-bisphosphate; HPLC, high pressure liquid chromatography; GFP, green fluorescent protein.

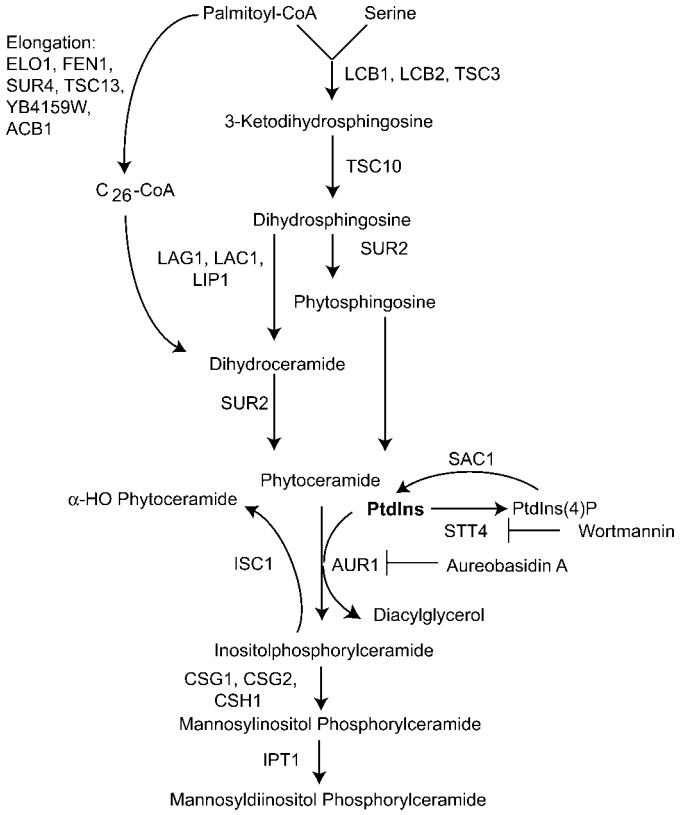

FIGURE 1. **Partial overview of sphingolipid and phosphatidylinositol metabolic pathways.** Phosphatidylinositol plays a central role as a substrate in both sphingolipid and phosphatidylinositol phosphate metabolism through Stt4p.

proposed to regulate sphingolipid levels by influencing ceramide synthesis and Isc1p activity (Refs. 8–11 and reviewed in Ref. 21). However, mechanistic data are largely lacking. The effects on sphingolipids of abrogating Sac1p activity have not previously been evaluated.

Although mechanistic information is scarce, a few studies provide hints that PtdIns metabolism may influence sphingolipid levels through substrate level interactions. For example, the IPC synthase Aur1p consumes PtdIns by transfer of its inositol phosphate head group to phytoceramide, forming IPC and diacylglycerol (15). The addition of inositol to the culture media decreased ceramide levels as IPC and, it was inferred, PtdIns increased (22); however, these effects have not been demonstrated in cells with perturbed PtdIns metabolic pathways. Furthermore, it has also been shown that inhibition of IPC synthesis increased the immediate metabolic precursor, ceramides (23), but the effect on other sphingolipids, including signaling sphingolipids, remains unclear. Together, these results raise tantalizing possibilities about the potential for turnover between PtdIns(4)P and PtdIns to regulate sphingolipid synthesis through nonsignaling mechanisms.

We undertook the current study to determine whether modulating turnover of the pool of PtdIns(4)P regulated by Sac1p and Stt4p significantly impacts sphingolipid metabolism. Data indicated that deletion of SAC1 increased levels of sphingoid bases without increasing *de novo* sphingolipid synthesis. Additionally, phytoceramide and sphingoid base phosphate levels were also elevated. In contrast to these effects, deletion of SAC1 decreased complex sphingolipids, whereas inhibition of Stt4p with wortmannin increased them. Deletion of SAC1 decreased PtdIns levels dramatically, whereas acute inhibition of Stt4p increased PtdIns, indicating that these effects may occur via substrate supply. Additionally, it was shown that treatment with wortmannin reduced sphingoid bases, and inhibition of IPC synthase overcame this decrease, supporting IPC synthesis as the point of interaction. These data support a substrate level metabolic interaction between sphingolipid levels and PtdIns(4)P turnover by Sac1p and Stt4p; this interaction appears to occur independently of recently proposed PtdIns(4,5)P_2 effector signaling. Finally, using nutrient permease levels as a readout, it was demonstrated that these changes affect a biologically relevant pool of sphingoid bases.

TABLE 1
Yeast strains used in this study

Strain	Genotype
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
BY4742:*sac1Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sac1Δ::G418*
BY4742:*cnb1Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cnb1Δ::G418*
BY4742:*slm1Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 slm1Δ::G418*
BY4742:*slm2Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 slm2Δ::G418*

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—The yeast strains used in this study are shown in Table 1 with their genotypes. Single deletion mutants in the BY4742 background were obtained from the Euroscarf yeast deletion library. [^{3}H]Serine (ART 246) and [^{3}H]inositol (0116B) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Routine cultures were seeded from 5-ml overnight cultures into yeast-protease-dextrose medium, which contains 1% yeast extract, 2% proteose peptone, and 2% dextrose and grown in a water bath at 30 °C with shaking at 200–250 rpm. For routine experiments, excepting those using radioactivity, cultures were grown to mid-logarithmic phase and then aliquotted into 50-ml Falcon tubes and incubated in a water bath at 30 °C with shaking at 200–250 rpm. For heat stress, the tubes were transferred to an identical water bath at 39 °C with shaking at 200–250 rpm. Wortmannin was purchased from Calbiochem (La Jolla, CA), and aureobasidin A was purchased from Takara Bio USA (Madison, WI). The cells were treated for 10 min with wortmannin or 20 min with aureobasidin A. Myriocin was purchased from Sigma-Aldrich. The cells were treated for 5 or 15 min with myriocin, as indicated.

Sphingoid Base Measurements—After treatment as described, the cells were collected by centrifugation at 3500 × *g* at room temperature for 3–5 min followed by resuspension in 3 ml of 2:1 chloroform:methanol. Lipids were extracted by the method of Bligh and Dyer (24). One-third of the sample was used for total lipid phosphate determination as described previously (25). The remaining two-thirds were subjected to mild alkaline hydrolysis and resolved by HPLC as described previously (26). The peaks were quantified based on signal derived from an internal standard (L-threo-phytosphingosine).

Labeling Studies—For pulse labeling studies, the cells were grown to mid-logarithmic phase, harvested by centrifugation, and resuspended in yeast-protease-dextrose medium from

the original culture. Wortmannin (10 μg/ml) was added immediately prior to labeling as indicated. [^{3}H]Inositol (20 μCi/ml) was added. Aliquots of 0.5 ml were taken after 10 min and pipetted into five volumes of ice-cold 10% trichloroacetic acid solution. Lipids were extracted and resolved by TLC in a solvent system of chloroform:methanol:NH_4OH (9:7:2) as described previously (27). For steady-state labeling, the cells were grown to saturation and seeded in SD complete medium containing 1 μCi/ml [^{3}H]inositol. The cells were grown for 21 h to mid-logarithmic phase. As indicated above, aliquots of 0.5 ml were taken, and lipids were extracted and resolved by TLC. The bands visualized by this method were scraped and quantified by liquid scintillation.

Liquid Chromatography-Mass Spectrometry Measurements—After treatment as described, the cells were collected by centrifugation at 3500 $\times$ *g* at room temperature for 3–5 min, snap-frozen in an methanol/solid CO_2 bath, and analyzed as described previously (28).

Western Blot—The cells were grown to mid-logarithmic phase, treated as specified, harvested by centrifugation, flash-frozen, and stored at −80 °C until ready for protein isolation. Then cells were washed in 20 ml of sterile water, pelleted, resuspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, containing 40 μl/ml protease inhibitor mixture from Roche Applied Science. Glass beads were added to resuspended pellets, and the cells were vortexed for four periods of 30 s intercalated with 30 s on ice. The extract was centrifuged at 13,000 rpm for 10 min, and the supernatant was removed. Additional buffer was added to the pellet, and the cells were again vortexed as above. The extracts were combined, and protein concentration was measured by BCA analysis using the Pierce Micro BCA protein assay kit from Fisher. Protein was adjusted to ~2 μg/μl, and the samples were boiled and subsequently analyzed by Western blot using 10 μg of total protein/lane, as described previously (29). The membranes were incubated with a rabbit polyclonal anti-GFP antibody (1:1000 dilution; Santa Cruz Biotechnology) for 2 h at room temperature, washed three times with phosphate-buffered saline, and incubated for 1 h with goat anti-rabbit IgG-HRP (1:5000 dilution; Santa Cruz Biotechnology). They were again washed three times in phosphate-buffered saline and then visualized using ECL reagents (Amersham Biosciences).

RESULTS

Deletion of SAC1 Increases Sphingoid Bases—Previous reports have demonstrated changes in sphingolipid metabolism accompanying perturbation of PtdIns metabolism (11). Additionally, phenotypic overlaps between sphingolipid and phosphoinositide metabolism mutants may indicate a functional connection. For example, altered sphingoid base levels result in defects in endocytosis and actin organization (29, 30); deletion of the PtdIns(4)P-phosphatase Sac1p also causes defects in these processes (20). To determine whether sphingolipid metabolism was altered by perturbation of PtdIns(4)P pools, sphingolipids were measured in a *sac1*Δ strain, which exhibits a 7–10-fold increase in PtdIns(4)P (20, 31). Deletion of SAC1 increased C_{18} PHS over 5-fold compared with wild type cells and also significantly increased C_{20} species (Fig. 2). These data suggested that preventing Sac1p-mediated hydrolysis of PtdIns(4)P to PtdIns increased sphingoid base levels.

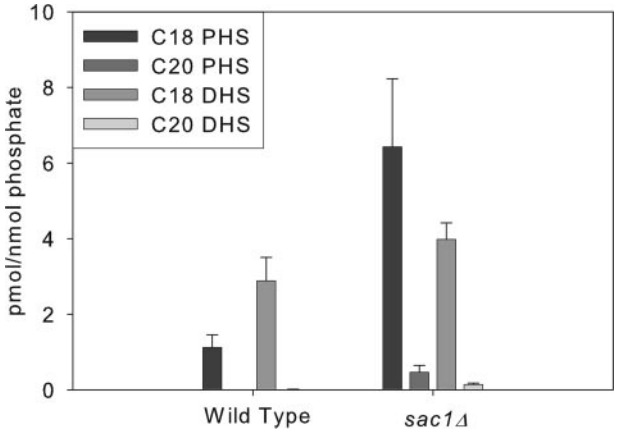

FIGURE 2. **Effect of *sac1*Δ on C_{18} and C_{20} phyto- and dihydrosphingosine levels.** The cells were grown at 30 °C in rich medium to mid-logarithmic phase. Aliquots of 10 ml were transferred to 50-ml tubes and grown for 5 min. The cells were harvested by centrifugation, and the lipids were extracted and analyzed by HPLC as described under "Materials and Methods." The data presented are the averages ± S.E. of three independent experiments performed in triplicate.

Deletion of SAC1 Does Not Increase de Novo Sphingoid Base Synthesis—The increase in bases in *sac1*Δ might be attributed to either increased *de novo* synthesis or decreased conversion into downstream metabolites. Two complementary approaches were used to determine whether *de novo* synthesis was the source of the accumulation of bases observed in *sac1*Δ.

To assess whether the increased sphingoid bases in *sac1*Δ resulted from increased *de novo* synthesis, sphingoid bases were measured in cells treated with myriocin, a selective inhibitor of serine palmitoyltransferase (32). In the wild type, a 15-min treatment with myriocin reduced basal sphingoid bases by 65% with respect to wild type control levels (a reduction of 2.45 pmol/nmol phosphate) (Fig. 3*A*). Moreover, a 5-min shift to the heat stress temperature of 39 °C, which is known to increase sphingoid bases via *de novo* synthesis, increased total bases to 257% of controls, and this increase was completely attenuated by myriocin treatment (total bases 66% of control) (Fig. 3*B*). In contrast, myriocin demonstrated relatively little effect in *sac1*Δ, with sphingoid base levels reduced by only 16% of mutant controls after 15 min (a reduction of 1.74 pmol/nmol phosphate) (Fig. 3*A*). Because *sac1*Δ mutants maintained elevated base levels after inhibition of *de novo* sphingoid base synthesis, this suggests that the elevated bases derive from an alternate mechanism.

A second approach was used to verify this result. Previous work has shown that the mechanism of increasing *de novo* sphingolipid synthesis during heat stress involves an increased rate of uptake of media serine, which was necessary and sufficient to drive *de novo* sphingoid base production (33). Intriguingly, the downstream PtdIns(4,5)P_2 effectors Slm1 and Slm2 colocalize with Pma1 (34), which regulates nutrient uptake through modulation of the proton gradient at the plasma membrane (35). Therefore, it was possible that deletion of the PtdIns(4)P 4-phosphatase may increase serine uptake indirectly and, consequently, increase sphingoid base levels. However, measurement of serine uptake demonstrated the opposite

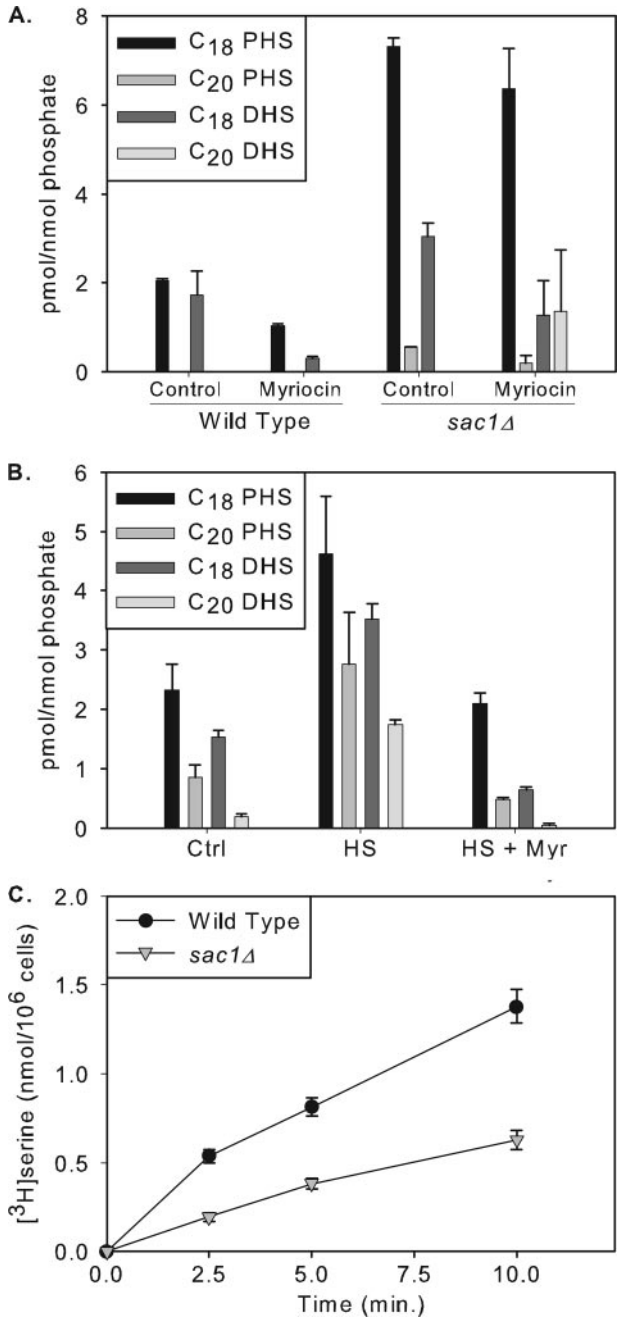

FIGURE 3. ***De novo* sphingoid base synthesis is not elevated in *sac1*Δ.** The cells were grown at 30 °C in rich medium to mid-logarithmic phase. *A*, aliquots of 10 ml were transferred to 50-ml tubes and grown for 15 min with 1 μM myriocin or vehicle. The cells were harvested by centrifugation, and the lipids were extracted and analyzed by HPLC as described under "Materials and Methods." The data presented are the averages ± S.E. of three independent experiments performed in triplicate. *B*, aliquots of 10 ml were transferred to 50-ml tubes. The cells were maintained at 30 °C or shifted to 39 °C and grown for 5 min with 1 μM myriocin or vehicle. The cells were harvested by centrifugation, and lipids were extracted and analyzed by HPLC as described under "Materials and Methods." The data presented are the averages ± S.E. of three independent experiments performed in triplicate. *C*, cells were spun down, washed with assay buffer (sodium citrate supplemented with 2% d-glucose), and resuspended in assay buffer. The cultures were placed in a 30 °C water bath and allowed to equilibrate for 1–2 min. [^{3}H]serine was added to the medium, and the aliquots were taken at the indicated times. Serine uptake was quantified as described under "Materials and Methods." The data presented are the averages ± *S.E.* of three independent experiments performed in triplicate. *HS*, heat stress; *myr*, myriocin.

effect. Specifically, the rate of uptake of [^{3}H]serine in *sac1*Δ was only 64% of the rate in wild type cells (0.215 ± 0.015 nmol serine·10^6 cells^{-1}·min^{-1} in wild type *versus* 0.077 ± 0.009 nmol serine·10^6 cells^{-1}·min^{-1} in *sac1*Δ) (Fig. 3*C*). These data may actually suggest a reduced rate of *de novo* sphingolipid synthesis in *sac1*Δ and, in addition to data in Fig. 3*A*, indicate that *de novo* synthesis does not likely mediate the increase in sphingoid bases observed in *sac1*Δ.

Deletion of SAC1 Increases Phytoceramide and Sphingoid Base Phosphates—Because elevation of sphingoid bases in the *sac1*Δ strain did not appear to occur through *de novo* synthesis, we hypothesized that the increase in bases may result from reduced incorporation into their downstream metabolites, sphingoid base phosphates, and ceramides. Previous data indicate that inhibiting the production of individual sphingolipids can increase concentrations of neighboring metabolites. Specifically, inhibition of ceramide synthesis by *lag1*Δ and *lac1*Δ increased sphingoid base phosphates, and deletion of sphingoid base kinases increased sphingoid bases and ceramides.[3] To determine the impact of *sac1*Δ on total sphingolipid profiles, lipid extracts were used for high throughput lipidomic analysis as previously described. These data indicated that, in contrast to decreased levels of downstream metabolites, *sac1*Δ produced little change in dihydroceramide and increased phytoceramide to 182% of wild type (Fig. 4*A*). Additionally, dihydrosphingosine-1-phosphate increased 27-fold, and phytosphingosine-1-phosphate increased 8-fold (Fig. 4*B*). These data indicate that the mechanism for increased sphingoid bases in *sac1*Δ does not likely involve inhibition of incorporation into immediate downstream metabolites (*i.e.* ceramides and/or base phosphates) and indicated an alternate mechanism for the observed sphingoid base increase.

Deletion of SAC1 Decreases Complex Sphingolipids—In yeast, ceramides serve as a substrate for inositol phosphorylceramide (IPC) synthesis through Aur1p. IPC then undergoes modification with mannose and an additional inositol phosphate group to generate mannosylinositol phosphorylceramide and mannosyldiinositol phosphorylceramide (36). Thus, the dramatic elevation in phytoceramide levels suggested that there may be an increase in complex sphingolipids. To test this hypothesis, the cells were grown for 21 h in the presence of [^{3}H]inositol, and complex sphingolipids were extracted, resolved by thin layer chromatography, and quantified by liquid scintillation. Surprisingly, despite robust increases in sphingoid bases, sphingoid base phosphates, and ceramides (Figs. 2 and 4), complex sphingolipid levels decreased dramatically in *sac1*Δ. In *sac1*Δ cells, IPC levels were 20% of wild type, mannosylinositol phosphorylceramide levels were 32% of wild type, and mannosyldiinositol phosphorylceramide levels were 38% of wild type (Fig. 5*A*). This steady-state labeling study indicates a reduction in complex sphingolipid mass in *sac1*Δ and may suggest a slower rate of complex sphingolipid synthesis in this mutant. Therefore, a pulse labeling strategy was utilized to determine whether *sac1*Δ decreased the rate of flux through complex sphingolipid synthesis. The cells were incubated for 10 min in the presence of

[3] A. L. Cowart and Y. A. Hannun, unpublished results.

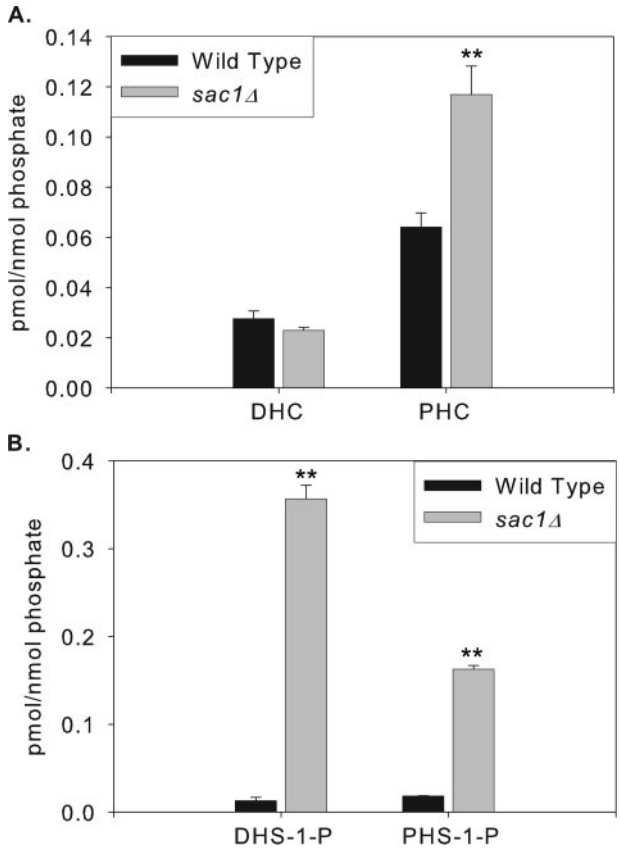

FIGURE 4. **Partial sphingolipid profile of *sac1*Δ mutants.** The cells were grown to mid-logarithmic phase in yeast-protease-dextrose medium. The sphingolipids were extracted and measured by liquid chromatography-mass spectrometry and normalized to total phospholipids as described under "Materials and Methods." Total lipid levels inclusive of all chain lengths are given for dihydroceramide (*DHC*, *A*) and phytoceramide and dihydrosphingosine-1-phosphate (*DHS-1-P*) and phytosphingosine-1-phosphate (*PHS-1-P*, *B*). The results are shown as the averages of three independent cultures ± S.E. **, $p < 0.01$ respective to control.

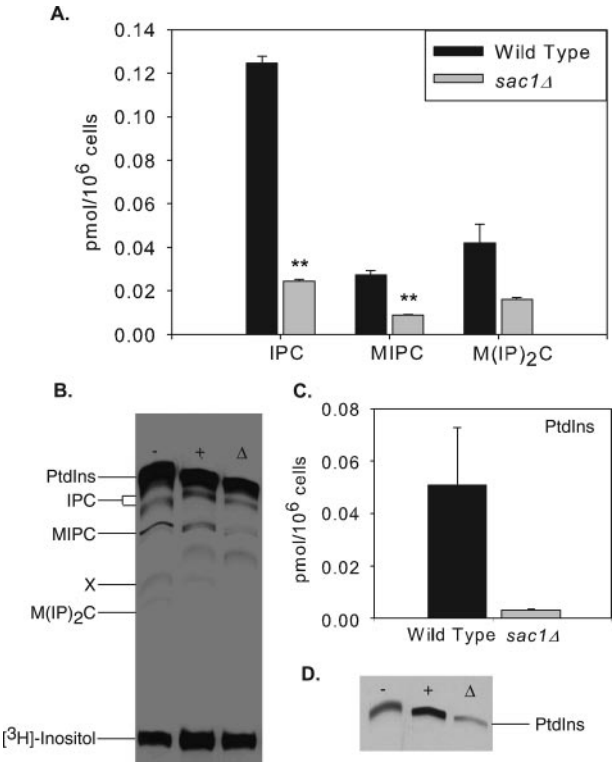

FIGURE 5. **Effects of *sac1*Δ and wortmannin on PtdIns and PtdIns-containing sphingolipids.** For *A* and *C*, the cells were grown to saturation and inoculated into SD medium containing [^{3}H]inositol. They were grown for 21 h to mid-logarithmic phase. After harvesting, the lipids were extracted and analyzed by TLC. Film was exposed to the TLC plates for 48 h. The bands visualized by TLC were scraped and counted by liquid scintillation, and molar quantities were calculated based on the proportion of radiolabeled inositol in the medium. The results are the averages of three independent cultures ± S.E. **, $p < 0.01$ respective to control. For *B* and *D*, the cells were grown to mid-logarithmic phase in yeast-protease-dextrose medium and resuspended in SD medium. Some were pretreated with 10 μg/ml wortmannin for 5 min as indicated. [^{3}H]Inositol was added, and the cells were incubated for 10 min. After harvesting, the lipids were extracted and analyzed by TLC. Film was exposed to the TLC plates for 16 h (*B*) or 3 h (*D*). −, control; +, wortmannin, Δ, *sac1*Δ.

[^{3}H]inositol, and lipids were extracted and resolved as described under "Materials and Methods." As in the steady-state labeling experiment, pulse labeling demonstrated a decrease in complex sphingolipids in *sac1*Δ (Fig. 5*B*), indicating that elimination of Sac1p activity decreased both the flux and mass of complex sphingolipids.

Inhibition of Stt4p Increases Flux through Complex Sphingolipids—The lipid phosphatase Sac1p primarily targets the pool of PtdIns(4)P produced by Stt4p (20). To determine whether the decrease in complex sphingolipids in *sac1*Δ results from altered turnover of PtdIns(4)P/PtdIns, cells were treated with the Stt4p inhibitor wortmannin (19), which has been demonstrated to decrease PtdIns(4)P. The cells pulse-labeled with [^{3}H]inositol in the presence of wortmannin increased both IPC and mannosylinositol phosphorylceramide relative to untreated controls (Fig. 5*B*). Thus, inhibition of Stt4p, which decreases consumption of PtdIns for PtdIns(4)P synthesis, increased complex sphingolipids. Conversely, deletion of SAC1, which decreases regeneration of the PtdIns pool and increases PtdIns(4)P (20), decreased complex sphingolipids. Together, these results suggest that PtdIns/PtdIns(4)P dynamics through Stt4p and Sac1p impact complex sphingolipid synthesis, which may occur through modulation of PtdIns available for complex sphingolipid synthesis.

Deletion of SAC1 Reduces PtdIns Levels—Because complex sphingolipid synthesis requires PtdIns as a substrate (15), regulation of PtdIns levels provides one potential mechanism by which Sac1p may affect complex sphingolipid synthesis (Fig. 1). Indeed, previous data indicate that deletion of SAC1 decreased PtdIns (37). Supporting the hypothesis of a substrate level effect, steady-state labeling revealed that the *sac1*Δ strain, which harbors reduced complex lipids, showed labeled PtdIns levels at only 6.0% of wild type (Fig. 5*C*). During pulse labeling, *sac1*Δ decreased PtdIns by 31% of wild type, whereas treatment with the Stt4p inhibitor wortmannin increased PtdIns to 169% of wild type (Fig. 5*D*). These data suggest that dynamics of the Sac1p/Stt4p-mediated pool of PtdIns(4)P significantly impact cellular PtdIns levels, which raised the possibility that they impact complex sphingolipid synthesis by limiting PtdIns substrate available to Aur1p.

Inhibition of IPC Synthase Ameliorates Reduction in Sphingoid Bases by Wortmannin—The data strongly suggested that deleting SAC1 reduced the PtdIns available for complex sphingolipid syn-

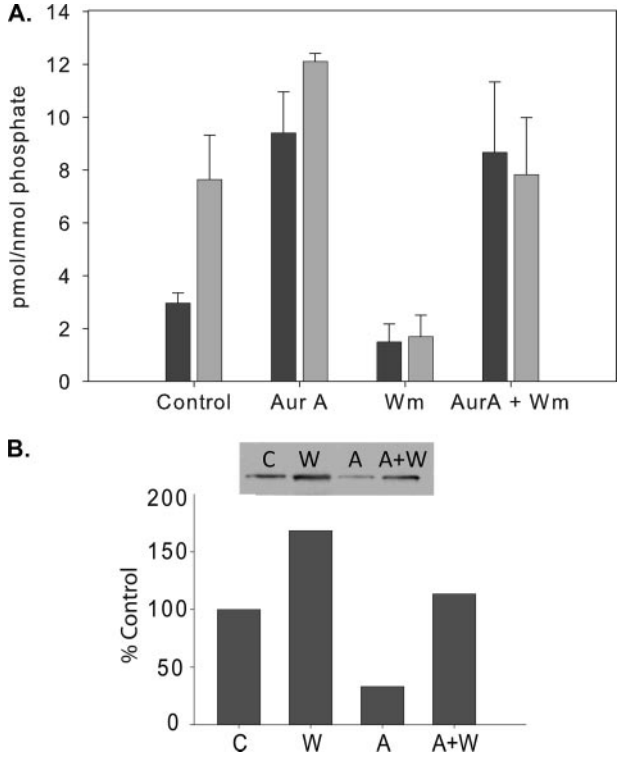

FIGURE 6. **Aureobasidin A abrogates the effects of wortmannin on sphingoid bases and Agp2-GFP protein levels.** The cells were grown at 30 °C in rich medium to mid-logarithmic phase. *A*, cells were pretreated with wortmannin or with aureobasidin A and grown for 5 min. The cells were harvested by centrifugation, and lipids were extracted and analyzed by HPLC as described under "Materials and Methods." The *dark gray bars* represent C_{18} phytosphingosine; the *light gray bars* represent C_{18} dihydrosphingosine. The data presented are the averages ± S.E. of two to three independent experiments performed in triplicate. *Aur A*, aureobasidin A; *Wm*, wortmannin. *B*, cells were harvested by centrifugation and snap frozen. Protein was extracted, and immunoblotting was carried out as described under "Materials and Methods." The blot shown is representative of two blots. *C*, control; *W*, wortmannin; *A*, aureobasidin A.

thesis. This concept is supported by the literature, which indicates that sphingolipid metabolism represents a highly interconnected network. In previous studies, treatment with aureobasidin A, which inhibits the IPC synthase Aur1p (38), increased ceramides while reducing levels of diacylglycerol, the other product of the IPC synthesis reaction (23). Furthermore, the addition of inositol to the culture medium markedly increased cellular PtdIns (39), resulting in increased complex sphingolipid synthesis with concomitant decreases in ceramides (22). This suggested the hypothesis that attenuating complex sphingolipid synthesis could cause an accumulation not only of the immediate metabolic precursor, ceramide, but also the effects could extend to the precursors of ceramides, the sphingoid bases. To test whether inhibition of complex sphingolipid synthesis would in fact increase bases, cells in logarithmic phase were treated with aureobasidin A, which attenuates IPC synthesis. This treatment increased levels of C_{18} PHS and DHS more than 3-fold (Fig. 6*A*), indicating that, indeed, attenuation of IPC synthesis caused accumulation of sphingoid bases, and raised the hypothesis that the effects on sphingoid bases in the *sac1*Δ mutant derived from decreased complex sphingolipid synthesis.

This hypothesis also implies that limiting conversion of PtdIns to PtdIns(4)P, the substrate of Sac1p, which increased PtdIns (Fig. 5*D*), would affect sphingoid bases in the opposite manner of *sac1*Δ. To test this hypothesis, wortmannin, an inhibitor of the PtdIns 4-kinase Stt4p (19), was used to reduce levels of PtdIns(4)P. As predicted, acute inhibition of Stt4p activity reduced C_{18} PHS and DHS to 30% of the control level (Fig. 6*A*). It is unlikely that this reduction is due to reduced *de novo* sphingolipid synthesis, because serine uptake, which is both necessary and sufficient to drive *de novo* sphingoid base synthesis (33), increased to 139% of control with acute wortmannin treatment (supplemental Fig. S1).

If wortmannin treatment decreased sphingoid bases by increasing their conversion into ceramide and, subsequently, IPC, then cotreatment with aureobasidin A, which would prevent IPC synthesis, would be expected to abrogate the effects of wortmannin on sphingoid bases. Indeed, inhibiting utilization of PtdIns for IPC synthesis overcame the wortmannin-induced sphingoid base defect, and sphingoid bases remained at normal to elevated levels (Fig. 6*A*). These data further support the hypothesis that changes in PtdIns(4)P metabolism affect sphingolipid levels at least in part by altering availability of PtdIns as a substrate for Aur1p, and, moreover, that attenuation of complex lipid synthesis either through decrease in PtdIns or through inhibition using aureobasidin A leads to an increase in sphingoid bases.

Inhibition of Stt4p Increases Nutrient Permease Levels—Because of the major defects in sphingoid base regulation in cells lacking Sac1p or Stt4p activity, it seemed likely that these cells would also harbor aberrant sphingoid base-related phenotypes. A well established function of sphingolipids is the modulation of the levels of amino acid transporters at the plasma membrane (29, 30). Specifically, previous studies demonstrated that elevated levels of PHS inhibited nutrient import (30) and activated ubiquitin-dependent proteolysis of the uracil permease Fur4 (29). To determine the effect of altered PtdIns(4)P metabolism on permease levels, protein levels of the amino acid permease Agp2-GFP were measured. Because of potential compensatory changes in cells with a constitutive defect such as *sac1*Δ, protein levels were not measured in the mutant; instead, the levels were measured after acute inhibition of Stt4p or Aur1p. Strikingly, acute treatment with the Stt4p inhibitor wortmannin elevated levels of Agp2-GFP to 168% of control. On the other hand, treatment with aureobasidin A, which inhibits IPC synthesis and increases sphingoid bases, reduced protein levels to 33% of control. Cotreatment with aureobasidin A and wortmannin resulted in protein levels that were similar to control (113% of control) (Fig. 6*B*). These data mirror the effects of these treatments on sphingoid bases and thus indicate that manipulation of the Stt4p/Sac1p-dependent pool of PtdIns(4)P causes sphingolipid-dependent biological effects. Moreover, these changes in nutrient permease levels may explain the unexpected serine uptake data observed in both *sac1*Δ and wortmannin treatment (Fig. 3*c* and supplemental Fig. S1, respectively).

DISCUSSION

Although both anabolic and catabolic pathways of sphingolipid metabolism are well understood, mechanisms regulating these pathways or controlling flux through them remain obscure. At the same time, new roles are emerging for PtdIns, its downstream phosphorylated metabolites, and their effectors (4, 6, 8, 11, 34, 40, 41). Several lines of evidence, largely reliant on yeast genetic studies, suggest regulation of sphingolipid

metabolism by phosphoinositides; however, no consensus has emerged about either the nature of the regulatory relationships or the mechanisms by which they occur (8–11). The purpose of this study was to determine whether substrate level interactions between the Sac1p/Stt4p pathway and Aur1p serve as a major mechanism by which PtdIns(4)P metabolism modulates sphingolipid levels.

Our data demonstrate for the first time that deletion of the gene for the lipid phosphatase SAC1 resulted in elevated sphingoid bases (Fig. 2). This accumulation did not result from increased *de novo* synthesis, suggesting that reduced utilization of bases for downstream metabolites may cause the observed elevation of sphingoid base levels. Deletion of SAC1 also increased levels of phytoceramide and, dramatically, sphingoid base phosphates (Fig. 4). In contrast, complex sphingolipids were reduced in *sac1*Δ during both steady-state and pulse labeling (Fig. 5, *A* and *B*), indicating a reduction in both the mass of complex sphingolipids and the rate of their synthesis. PtdIns levels were reduced during both long term and pulse labeling experiments (Fig. 5, *C* and *D*), suggesting that the effects on sphingolipids may be due to reduced PtdIns availability for complex sphingolipid synthesis through Aur1p. Indeed, inhibition of Aur1p replicated the increase in sphingoid bases seen in *sac1*Δ mutants and overcame the decrease in bases observed in cells treated with the Stt4p inhibitor wortmannin (Fig. 6*A*). This further supports the hypothesis that these effects occur at the point of Aur1p-mediated inositol phosphosphingolipid synthesis.

Previous studies indicate that sphingolipid metabolism represents a highly interconnected network, and inhibiting production of many sphingolipid metabolites increases concentrations of their precursors or neighboring metabolites. The idea that a reduction in Aur1p activity can impact levels of neighboring metabolites has been explored extensively. However, to our knowledge, this is the first study to demonstrate that metabolic effects of modulation of IPC synthesis can extend beyond neighboring metabolites and upstream to sphingoid bases and sphingoid base phosphates.

Consistent with these principles, deletion of SAC1 reduced PtdIns and complex lipids while increasing upstream sphingolipid metabolites. Conversely, inhibition of Stt4p with wortmannin increased PtdIns and complex sphingolipids while decreasing sphingoid bases. The wortmannin-induced decrease in sphingoid bases was overcome by inhibition of IPC synthesis, further supporting a model in which modulation of the Stt4p/Sac1p-dependent pool of PtdIns(4)P influences sphingoid base levels via substrate supply to Aur1p. Specifically, we propose a model whereby deletion of SAC1 causes PtdIns to become sequestered as PtdIns(4)P, resulting in decreased PtdIns available for Aur1p-mediated complex sphingolipid synthesis. This block in complex sphingolipid synthesis then causes an accumulation of the metabolic precursor for IPC synthesis, ceramide, and its metabolic precursors, sphingoid bases.

One challenge to this model comes in the alternative hypothesis that Isc1p, which catabolizes IPC to generate ceramides (42), caused the changes in complex lipid levels. However, levels of α-HO phytoceramide, the product of Isc1p (43), are reduced to 27% of wild type in *sac1*Δ (supplemental Fig. S2). Indeed, the overall ceramide profile in *sac1*Δ mutants (Fig. 4*a* and supplemental Fig. S2) does not reflect the whole cell profile expected for cells with elevated Isc1p activity (43), discounting the idea that Isc1p activity decreased complex sphingolipids in this mutant. Additionally, altered Isc1p activity would not explain the dramatic decrease in PtdIns observed in *sac1*Δ (Fig. 5, *C* and *D*). Together, these data support Aur1p, rather than Isc1p, as the key enzyme in this interaction.

One mechanism by which deletion of SAC1 may limit substrate availability to IPC synthase is the direct sequestration of PtdIns as PtdIns(4)P. Previously, it has been shown that dramatic alterations in phosphoinositide profiles occur when PtdIns(4)P turnover is perturbed with *sac1*Δ; indeed, the proportion of PtdIns(4)P increases to 12–24% of labeled phosphoinositides, a 7–10-fold change (20, 31). The K_m of Aur1p is 5 mol% PtdIns (44), and the concentration of PtdIns in the cell is 4.6 mol% during the exponential growth phase (45). The magnitude of these changes lends credence to the notion that altering PtdIns(4)P levels can modulate PtdIns to an extent that would significantly impact complex sphingolipid synthesis. Additionally, the reduced turnover of PtdIns(4)P to PtdIns is thought to contribute significantly to the inositol auxotrophy of *sac1*Δ mutants (46); this further supports the idea that changes in Sac1p/Stt4p function can directly alter cellular PtdIns levels. In this model, decreased turnover of PtdIns(4)P to PtdIns by *sac1*Δ would reduce complex sphingolipid synthesis by sequestering PtdIns in PtdIns(4)P and downstream metabolites; this would result in a buildup of ceramides and sphingoid bases.

In contrast to the mechanism proposed here, several recent studies have described regulation of sphingolipid metabolism by PtdIns(4,5)P_2 effectors, particularly through Slm1/2p and calcineurin (8, 10, 11). Significantly, total sphingoid base levels were virtually unaltered in single-deletion mutants for SLM1, SLM2, and the calcineurin regulatory subunit CNB1 (supplemental Fig. S3), suggesting that the effect on sphingoid bases in *sac1*Δ does not occur primarily through known PtdIns(4,5)P_2-dependent signaling pathways. Additionally, other observations in *sac1*Δ mutants and wortmannin-treated cells do not correspond to the described effects of PtdIns(4,5)P_2 effector-mediated regulation. For example, it has been proposed that Slm1/2p regulate IPC levels by inhibiting Isc1p, which catabolizes IPC (11). Thus, a reduction in PtdIns(4,5)P_2 levels consequent to Stt4p inhibition would be expected to relieve the inhibition of Isc1p by Slm1/2p, decreasing complex sphingolipid levels. However, acute wortmannin treatment was shown to increase these levels (Fig. 5*B*). Additionally, a previous study demonstrated very little change in PtdIns(4,5)P_2 levels in *sac1*Δ (31), making altered Slm1/2p activity an unlikely source of its effects. Likewise, it has been proposed that calcineurin inhibits ceramide synthesis (9) and that calcineurin is itself inhibited by Slm1/2p (11). Wortmannin-treated cells, which produce less PtdIns(4,5)P_2, thus would be thought to have less Slm1/2p activity. This would increase calcineurin activity, leading to reduced ceramide synthesis and, most likely, reduced complex sphingolipids and increased sphingoid bases. However, this is the opposite of what is observed in Stt4p-inhibited cells (Figs. 5*C* and 6*A*). The corresponding predictions for *sac1*Δ similarly fail to describe the data obtained in these studies. Thus, although PtdIns(4,5)P_2 effector pathways provide an important

mode of sphingolipid regulation, the effects described in the present study occur through an independent mechanism.

A key question for this study is whether the changes in sphingolipid levels, especially sphingoid bases, have any biological relevance in the cell. One established readout of sphingoid base-mediated effects involves nutrient permeases. Specifically, increased phytosphingosine reduces nutrient import (30) and levels of nutrient permeases (29, 30). Intriguingly, serine uptake was reduced in *sac1*Δ mutants and increased in wortmannin-treated cells. Indeed, *sac1*Δ mutants took up serine at 64% of the rate of wild type (Fig. 3*C*), whereas inhibition of Stt4p with wortmannin increased serine uptake to 139% of control (supplemental Fig. S1). This suggested the possibility that the drastic elevation of sphingoid bases in *sac1*Δ resulted in an increased rate of permease degradation, thus decreasing levels of permeases, whereas the reduced sphingoid bases observed upon inhibition of Stt4p would allow an increase in permease levels. To test this hypothesis, protein levels of the nutrient permease Agp2-GFP were measured by immunoblot in cells treated acutely with wortmannin or aureobasidin A. Consistent with this hypothesis, inhibition of Stt4p led to levels of Agp2-GFP that were 168% of control (Fig. 6*B*), whereas inhibition of IPC synthesis decreased protein levels to 33% of control (Fig. 6*B*). Inhibition of both Stt4p and Aur1p, which overcomes the effect of wortmannin on sphingoid bases, resulted in protein levels similar to control (113% of control). Potentially, this may indicate a negative feedback loop in which increased sphingoid base levels decrease serine uptake and thus reduce the rate of *de novo* sphingolipid synthesis; reduced sphingoid base levels would have the opposite effect. More concretely, these results suggest that alterations in sphingolipid levels by perturbation of PtdIns pathways affect biologically active pools of sphingoid bases. This implies that future studies perturbing key PtdIns-metabolizing enzymes should consider potential sphingolipid-mediated effects when interpreting phenotypic changes in these systems.

Topology also poses a significant issue in the resolution of the relationships between Stt4p, Sac1p, and complex sphingolipid metabolism. Indeed, Stt4p localizes to the plasma membrane (16), whereas Sac1p localizes primarily to the endoplasmic reticulum (20). The substrate level relationship between these two enzymes is well established (20), but the mechanism of PtdIns(4)P transport from the plasma membrane to the binding site of Sac1p remains unclear. Likewise, this study does not address the very important issue of transport between the PtdIns pool supplying Aur1p, which localizes to the Golgi (47), and those pools associated with Stt4p and Sac1p. However, sphingolipid metabolism is distributed across multiple organelles. Additionally, there are multiple known PtdIns transport proteins (48); this supports the possibility of phospholipid transport between cellular compartments for sphingolipid synthesis.

The decrease in complex sphingolipids in *sac1*Δ and increased complex sphingolipids with acute wortmannin treatment contrast data from a previous study that showed reduced complex sphingolipids in *stt4*^{ts} (11). However, in our hands the background strain from that study produced an unusual sphingolipid profile and a highly depressed heat stress response (data not shown). Additionally, the sphingolipid profile of the *stt4*^{ts} mutant was altered at permissive as well as restrictive temperature (Ref. 11 and data not shown). The constitutive reduction of function of Stt4p, which is a vital protein, may induce changes in sphingolipid levels via signaling through Slm1/2, calcineurin, and TORC2 (9, 11); these effects may not be as pronounced at early time points with acute inhibitor treatment and would not be predicted to occur in *sac1*Δ, in which PtdIns(4,5)P_2 levels remain virtually unchanged (31). Finally, to use temperature-sensitive mutants, Tabuchi *et al.* (11) evaluated sphingolipids under heat stress conditions. It has been shown extensively that sphingolipid levels are drastically altered in response to heat stress conditions (reviewed in Ref. 1), and lipid levels during heat stress may not correspond to what would be seen with the same disruptions under nonstress conditions.

Still unknown are possible substrate effects through other PtdIns kinases. However, it has previously been shown that dysfunction of the PtdIns-4-kinase Pik1p does not appear to affect sphingolipid levels (11). This suggests that consumption and regeneration of the Stt4p/Sac1p-dependent pool of PtdIns(4)P holds a unique importance for sphingolipid metabolism.

In conclusion, in addition to putative interplay between PtdIns(4,5)P_2 effectors and sphingolipid metabolism, the data indicate that Sac1p and Stt4p activity have substantial metabolic effects on sphingolipid levels via modulation of substrate supply to Aur1p. This substrate level effect occurs independently of known PtdInsP effector signaling and appears to outweigh signaling through Slm1/2p and calcineurin in this context. Finally, the present study provides an important caveat to the interpretation of experiments that interfere with phosphatidylinositol phosphate metabolism, and, in light of these data, it must be considered which phenotypes are due to altered sphingolipid levels and which ones are attributable to *bona fide* phosphatidylinositol phosphate signaling.

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