Orm protein phosphoregulation mediates transient sphingolipid biosynthesis response to heat stress via the Pkh-Ypk and Cdc55-PP2A pathways

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ABSTRACT Sphingoid intermediates accumulate in response to a variety of stresses, including heat, and trigger cellular responses. However, the mechanism by which stress affects sphingolipid biosynthesis has yet to be identified. Recent studies in yeast suggest that sphingolipid biosynthesis is regulated through phosphorylation of the Orm proteins, which in humans are potential risk factors for childhood asthma. Here we demonstrate that Orm phosphorality status is highly responsive to sphingoid bases. We also demonstrate, by monitoring temporal changes in Orm phosphorylation and sphingoid base production in cells inhibited for yeast protein kinase 1 (Ypk1) activity, that Ypk1 transmits heat stress signals to the sphingolipid biosynthesis pathway via Orm phosphorylation. Our data indicate that heat-induced sphingolipid biosynthesis in turn triggers Orm protein dephosphorylation, making the induction transient. We identified Cdc55–protein phosphatase 2A (PP2A) as a key phosphatase that counteracts Ypk1 activity in Orm-mediated sphingolipid biosynthesis regulation. In total, our study reveals a mechanism through which the conserved Pkh-Ypk kinase cascade and Cdc55-PP2A facilitate rapid, transient sphingolipid production in response to heat stress through Orm protein phosphoregulation. We propose that this mechanism serves as the basis for how Orm phosphoregulation controls sphingolipid biosynthesis in response to stress in a kinetically coupled manner.

INTRODUCTION Sphingoid intermediates, including sphingoid bases, sphingoid base phosphates, and ceramides (Figure 1), play important roles in regulation of cell growth, differentiation, senescence, and apoptosis. Serine palmitoyltransferase (SPT) mediates the rate-limiting first step in sphingolipid biosynthesis (Figure 1). Despite the importance of sphingoid intermediates as bioactive molecules, the regulation of sphingolipid biosynthesis through SPT is not well understood (Cowart and Hannun, 2007). A recent study revealed that yeast Orm proteins, encoded by ORM1 and ORM2, form a conserved complex with SPT and that their phosphorylation status affects sphingolipid production (Breslow et al., 2010; Figure 1). The authors proposed that sphingolipids feedback regulate Orm protein phosphorylation, thus mediating sphingolipid homeostasis (Breslow et al., 2010). However, several important questions related to this model need answers. For example, whether and which sphingolipid species affect Orm phosphorylation are not known. In addition, how temporal regulation of Orm phosphoregulation relates to dynamic changes in sphingolipid biosynthesis is not...
known. Answers to these questions are required to better understand Orm-mediated sphingolipid homeostasis.

Another important question concerns how Orm-mediated sphingolipid homeostasis may function in a physiological context. Increasing evidence suggests that various stimuli trigger accumulation of sphingoid intermediates, which in turn function as bioactive molecules mediating cellular responses (Hannun and Obeid, 2008; Dickson, 2010). For instance, heat-stress-induced sphingoid intermediates act as signaling molecules to induce cellular responses such as translation initiation of heat shock proteins, gene regulation, and cell cycle arrest (Dickson et al., 1997; Mao et al., 1999; Jenkins and Hannun, 2001; Cowart and Hannun, 2005; Cowart et al., 2010; Meier et al., 2006; Han et al., 2010). It is striking that several groups demonstrated that heat stress induces sphingolipid biosynthesis in a rapid and transient manner (Dickson et al., 1997; Jenkins et al., 1997; Jenkins, 2003; Wells et al., 1998; Mao et al., 1999; Krzypek et al., 1999), suggesting that biosynthesis of sphingoid intermediates in response to stresses requires precise temporal regulation. We hypothesize that the dynamic changes in sphingoid intermediates levels upon heat stress may be caused by and/or may lead to changes of Orm phosphorylation. If this is the case, heat stress could serve as a model system to address the mechanism of how Orm phosphoregulation functions in the response of sphingolipid biosynthesis to stress in general.

A recent study showed that yeast protein kinases 1 and 2 (Ypk1/2), the homologues of mammalian serum- and glucocorticoid-inducible kinase (Casamayor et al., 1999), directly phosphorylate Orm proteins in vitro (Roelants et al., 2011). This result suggests that Ypk kinase may regulate sphingolipid homeostasis through its phosphorylation of the Orm proteins. However, whether Ypk kinase activity indeed affects sphingolipid production has not been examined. Of interest, the Ypk kinases were previously considered to be downstream of heat-induced sphingolipid base accumulation (Sun et al., 2000; Friant et al., 2001; Liu et al., 2005; Hannun and Obeid, 2008; Dickson, 2010). Thus, testing for a possible role for Ypk kinase activity in the sphingolipid biosynthesis response to heat stress is an important goal.

Of importance, the Orm phosphorylation state is set not only by protein kinases, but also by phosphatases. A reasonable prediction is that the phosphatases acting on the Orm proteins are likely to be involved in and/or regulated by sphingolipid levels. Protein phosphatase 2A (PP2A) was identified as an attractive candidate for a ceramide-activated protein phosphatase in yeast (Nickels and Broach, 1996). Inactivation of the regulatory subunits (such as Cdc55) or the catalytic subunits of PP2A was reported previously to suppress the endocytic defects of a mutant with impaired SPT activity (Friant et al., 2000), suggesting that PP2A may be involved in sphingolipid production. Because PP2A was suggested previously to be involved in multiple cellular signaling pathways (Jiang, 2006), the challenge is to identify a specific relationship between Cdc55-PP2A and the Orm proteins for regulation of sphingolipid biosynthesis.

In this article, by addressing the questions just raised, we demonstrate how Orm phosphorylation regulation controls sphingolipid biosynthesis in response to heat stress in a kinetically coupled manner. We identify a signaling pathway by which the conserved pKhr-Ypk signaling cascade and Cdc55-PP2A facilitate rapid, transient sphingolipid production upon heat stress through precise regulation of Orm protein phosphorylation. Our study will therefore provide a foundation for future studies of sphingolipid-related responses to other stimuli.

RESULTS
Orm2 is the major Orm species in budding yeast

A 3XFLAG sequence was inserted at the 5’ end of the genes ORM1 and ORM2, which encode the two homologous yeast Orm proteins. Because Orm2 expression was at least 10 times higher than Orm1 expression (Supplemental Figure S1a) and because 3XFLAG-Orm1 in an orm2Δ strain, but not 3XFLAG-Orm2 in an orm1Δ strain, caused slow cell growth (Supplemental Figure S1, b and c), we concluded that Orm2 is the major Orm protein. Consistently, orm2Δ cells but not orm1Δ cells showed perturbation in sphingolipid homeostasis (Han et al., 2010). These results suggested that Orm2 provides the majority of the Orm protein function. Thus, we decided to monitor Orm2 phosphorylation in our studies. As shown in Figure 2a, four groups of Orm2 bands were observed when resolved by phosphate-affinity gel, which is widely used for the analysis of phosphoprotein isotypes (Kinoshita et al., 2006). As previously reported (Breslow et al., 2010), the slower-migrating bands in the top three groups were confirmed to be phosphorylated forms of Orm2 (Supplemental Figure S2). On the basis of the principle responsible for protein separation on phosphate-affinity gels (Kinoshita et al., 2006), the bands in the top three groups are likely to be phosphorylated to different extents, with the slowest-migrating species being the most highly phosphorylated. The bands in the third group observed by Western blotting are often overexposed under the conditions that allow us to observe bands in all four groups (Figure 2a). Thus, we monitored changes in intensity of groups 1, 2, and 4 as clear indicators of changes in Orm2 phosphorylation status.

Exogenously provided sphingoid bases are sufficient to induce rapid Orm2 dephosphorylation

We examined how exogenously provided sphingoid bases, which are early sphingoid intermediates (Figure 1), affect Orm2 phosphorylation status. As shown in Figure 2, b and c, dephosphorylated Orm2, seen as the fastest-migrating bands (the fourth group), increased in

[schematic diagram of yeast sphingolipid biosynthesis from endogenous and exogenous precursors]

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**FIGURE 1:** Schematic diagram of yeast sphingolipid biosynthesis from endogenous and exogenous precursors. Myriocin is a potent inhibitor of SPT (Sun et al., 2000), which is the first and rate-limiting enzyme of the sphingolipid biosynthesis pathway (Bueed et al., 1991). Previous studies proposed that Orm proteins are dynamic negative regulators of SPT and their activities are inversely proportional to sphingolipid levels (Breslow et al., 2010). Exogenous sphingoid bases (DHS and PHS) can be converted into ceramides in the endoplasmic reticulum after phosphorylation and dephosphorylation by the indicated enzymes and can then be incorporated into complex sphingolipids (Qie et al., 1997; Nagiec et al., 1998; Funato et al., 2003).
Drosphingosine (DHS), another sphingoid base, had a similar effect on Orm2 dephosphorylation (unpublished data). Dephosphorylated Orm2 greatly increased within 2 min of treatment with 5 μM PHS at 25°C (Figure 2, d and e). In contrast, adding 5 μM sphingosine (Sph), which is a mammalian sphingoid base that cannot rescue a yeast sphingolipid deficiency (Wells and Lester, 1983), did not affect Orm2 dephosphorylation (Figure 2, d and f), indicating that the effect of PHS is highly specific. Furthermore, stearilamine (Jenkins and Hannun, 2001), which is a long-chain primary amine, also did not induce Orm2 dephosphorylation (unpublished data), providing further evidence for chemical specificity.

The rapid response to the exogenous addition of PHS (Figure 2e) suggests that the sphingoid base itself, rather than its downstream sphingolipid metabolites (Nagiec et al., 1997; Figure 1), triggers Orm2 dephosphorylation. To explore this possibility further, we used a knockout mutant of the LCB3 gene, which encodes the sphingosine-1P phosphatase required for ceramide synthesis from exogenous PHS (Mao et al., 1997, 1999; Qie et al., 1997; Figure 1). In the absence of Lcb3, exogenous PHS cannot be incorporated into ceramides (Qie et al., 1997; Funato et al., 2003). However, exogenous PHS still rapidly induced Orm2 dephosphorylation in the lcb3Δ strain (Figure 2g), suggesting that exogenous PHS induces Orm2 dephosphorylation without being converted to ceramides. A previous study indicated that lack of Orm2 results in increased PHS but decreased ceramides in vivo (Han et al., 2010). Here we found that Orm1 phosphorylation is greatly reduced in a strain lacking Orm2 (Supplemental Figure S1a), in agreement with PHS being sufficient to induce Orm dephosphorylation. In addition, exogenous PHS still triggered Orm2 dephosphorylation in mutant cells lacking both Lcb4 and Lcb5 (Figure 2h), which are sphingoid base kinases required for phosphorylation of exogenously provided PHS (Nagiec et al., 1998; Funato et al., 2003; Figure 1). Thus, exogenous PHS itself, without being converted to phosphorylated sphingoid bases (Figure 1), can induce Orm2 dephosphorylation. However, compared with the wild-type cells (Figure 2e), Orm2 dephosphorylation in lcb4Δ lcb5Δ cells responds to PHS to a lesser extent (Figure 2h), suggesting that phosphorylated sphingoid bases may also contribute to Orm2 phosphoregulation.

Taken together, our data demonstrate that exogenous addition of PHS is sufficient to rapidly induce Orm2 dephosphorylation, indicating thatOrm phosphorylation status is highly responsive to levels of sphingoid bases. Although we cannot exclude the possibility that ceramide and complex sphingolipids also

**FIGURE 2:** Exogenously provided sphingoid bases are sufficient to induce rapid Orm2 dephosphorylation. (a, b, d–h), Western blots showing Orm2 phosphorylation patterns after separation of the indicated yeast cell extracts on phosphate-affinity gels (top). P-Orm2 indicates phosphorylated forms of Orm2. We used 3-phosphoglycerate kinase 1 (Pgk1) as a loading control (bottom). All cells were grown to early log phase in YPD at 25°C before the indicated treatments. Cell extracts were prepared as described in Materials and Methods. (a) The majority of the Orm2 is moderately phosphorylated. Western blotting analysis of cell extracts from wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in media containing methanol alone (mock) or 5 μM PHS dissolved in methanol for the indicated times. (f) Exogenous sphingosine (Sph) does not affect Orm2 phosphorylation. Wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in the presence of 5 μM Sph (dissolved in methanol) for the indicated times. (g, h) Exogenous PHS induces rapid Orm2 dephosphorylation in lcb3Δ cells or in lcb4Δ lcb5Δ cells. lcb3Δ cells or lcb4Δ lcb5Δ cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in the presence of 5 μM PHS for the indicated times.

response to a 10-min treatment at 25°C with a natural yeast sphingoid base, phytosphingosine (PHS), in a concentration-dependent manner. PHS at 5 μM is sufficient to cause maximal effects on Orm2 dephosphorylation (Figure 2c), whereas 20 μM of exogenous dihydrosphingosine (DHS), another sphingoid base, had a similar effect.
We monitored Orm2 phosphorylation after cells were shifted from 25 to 39°C. Within 1 min after the temperature shift, dephosphorylated Orm2, which is the fastest-migrating band on phosphate-affinity gels, started to disappear (Figure 3a). Correspondingly, Orm2 phosphorylation levels increased, as evidenced by the increased abundance of the slower-migrating bands in the top two groups (Figure 3a). Orm2 phosphorylation further increased at 2 min (Figure 3a), with the maximal effect observed at ∼5 min, followed by a decrease (Figure 3, b and c). Orm2 phosphorylation was further reduced by 20 min of heat stress and then stabilized (Figure 3, b and c). Several independent studies demonstrated contribute to Orm phosphoregulation, the results with lcb3Δ mutants (Figure 2g) support the conclusion that neither of these sphingolipids is required for Orm phosphoregulation.

**Temporal association between Orm phosphorylation dynamics and de novo sphingoid base production in response to heat stress**

Previous studies suggested that heat stress induces rapid transient de novo accumulation of sphingoid intermediates, including PHS. We next asked whether and how Orm protein phosphorylation may be involved in heat-induced sphingolipid biosynthesis.

FIGURE 3: Orm2 phosphorylation dynamics and sphingoid base production upon heat shock. (a, b, d, e) Western blots showing Orm2 phosphorylation patterns after separation of the indicated yeast cell extracts on phosphate-affinity gels (top). P-Orm2 indicates phosphorylated forms of Orm2. Pgk1 was used as a loading control (bottom). Wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were used in all experiments and cultured to early log phase at 25°C before the indicated treatments. Cell extracts were prepared as described in Materials and Methods. (a–c) Temporal association between Orm phosphorylation dynamics and sphingoid base production upon heat stress. Cell extracts were prepared after cell cultures were shifted from 25 to 39°C for the indicated times. The relative intensity of the two slowest-migrating 3XFLAG Orm2 bands (groups 1 and 2) shown in b was quantified. Time-course experiments shown in b were performed an additional four times. Data collected from the five independent experiments were plotted in the graph shown in c (black line). Data shown represent the means with standard deviations. The C18-PHS concentration (c, gray, broken line) was determined by HPLC as described in Materials and Methods. (d), Orm2 phosphorylation status upon heat stress in the presence of exogenous sphingoid bases. Cells were shifted from 25 to 39°C for the indicated times. Methanol alone (control) or 5 μM PHS dissolved in methanol was added exogenously 4 min after cells were shifted from 25 to 39°C. (e) Orm2 phosphorylation upon heat stress in the presence of myriocin. Left, 0.5 μg/ml myriocin was added to cells upon shift from 25 to 39°C. Right, cells were treated with 0.5 μg/ml myriocin at 25°C for the indicated times.
that heat stress induces rapid de novo accumulation of various sphingoid intermediates, including sphingoid bases (Dickson et al., 1997; Jenkins et al., 1997; Jenkins, 2003; Wells et al., 1998; Mao et al., 1999; Skrzypek et al., 1999). The sphingoid base levels peak by 10–15 min and decrease to near-basal levels by 30 min of heat stress (Dickson et al., 1997; Jenkins et al., 1997). Our measurements of PHS levels confirmed these previous conclusions (Figure 3c, gray, broken line). Thus, our results demonstrate a striking temporal association between Orm phosphorylation dynamics and de novo sphingoid base production in response to heat stress (Figure 3c).

High levels of Orm phosphorylation induced by heat stress decrease when the sphingoid bases (PHS) reach their peak levels (Figure 3c, time points between 5 and 15 min), suggesting that heat-induced sphingoid base accumulation and Orm dephosphorylation may be mechanistically linked. To investigate this possibility further, we altered the timing of the sphingoid base accumulation by adding exogenous PHS 4 min after the temperature shift, when de novo–synthesized sphingolipids normally just begin to accumulate (Figure 3c; Dickson et al., 1997; Jenkins et al., 1997; Wells et al., 1998; Mao et al., 1999; Skrzypek et al., 1999). In control cells, increased Orm2 phosphorylation peaks after 4 min of heat stress and is only slightly diminished after 6 min of heat stress (Figure 3d, left). However, addition of exogenous PHS at 4 min reduced Orm2 phosphorylation to the basal level at 6 min (Figure 3d, right).

To further test whether de novo synthesis of sphingoid intermediates in response to heat stress is required to trigger Orm dephosphorylation, we monitored Orm2 phosphorylation in response to heat stress in the presence of myriocin, a potent SPT inhibitor (Sun et al., 2000; Figure 1). No obvious changes in Orm2 phosphorylation were observed up to 5 min after myriocin treatment at 25°C (Figure 3e), and 2 min of heat stress induced Orm2 phosphorylation irrespective of myriocin presence (Figure 3, b and e), indicating that heat stress affects Orm2 phosphorylation before the myriocin-sensitive step. Of interest, in the presence of myriocin, the increased Orm2 phosphorylation did not decline even after 30 min of heat stress (compare Figure 3e with Figure 3b), indicating that de novo synthesis of sphingoid intermediates is required for reducing the increased Orm2 phosphorylation caused by heat stress. Together, these results provide strong evidence that increased levels of sphingoid intermediates trigger Orm dephosphorylation during the heat stress response. Thus, our results suggest that both exogenously provided sphingoid intermediates (such as PHS) and the accumulation of physiologically induced sphingoid intermediates (by heat stress), cause rapid Orm2 dephosphorylation (Figures 2 and 3).

Ypk kinase transmits heat stress signals to the sphingolipid biosynthesis pathway via Orm phosphorylation

Another example of a striking temporal association between Orm phosphorylation and sphingolipid intermediate production is the observation that heat stress triggers Orm phosphorylation in <1 min (Figure 3a), with phosphorylation reaching its maximum before the level of sphingoid bases reaches its peak (Figure 3, b and c). This temporal association raises the important question of determining how the heat stress signal is transmitted to induce rapid Orm phosphorylation.

A recent study suggested that Ypk1, a homologue of mammalian serum- and glucocorticoid-inducible kinase (Casamayor et al., 1999), specifically phosphorylates Orm2 residues S46, S47, and S48 in vitro (Roelants et al., 2011). To examine the role of phosphorylation of these serine residues on Orm function, we mutated S46, S47, and S48 of Orm2 to alanine or aspartic acid to generate orm2-3A (mimicking the phosphorylated form) or orm2-3D (mimicking the phosphorylated form), respectively. The level of sphingoid bases was greatly reduced in orm2-3A cells and was greatly increased in orm2-3D cells (Supplemental Figure S3), providing evidence that phosphorylation of these serine plays a role in sphingolipid production. Furthermore, as shown in Figure 4a, the majority of orm2-3A protein is in dephosphorylated forms, demonstrating that phosphorylation of Orm2 residues S46, S47, and S48 is responsible for the slower migration of phosphorylated Orm2 (Figure 4a; Breslow et al., 2010). Together, these results strongly suggest that phosphorylation of Orm2 by Ypk1 is involved in Orm-mediated sphingolipid biosynthesis regulation.

To determine whether the rapid Orm2 phosphorylation induced by heat stress (Figure 3c, time points between 0 and 4 min) requires Ypk kinase activity in vivo, we generated an analogue-sensitive (Bishop et al., 2000) ypk1-as allele in ypk2Δ background. Deletion of YPK2 has no apparent phenotypic defect, but loss of YPK1 results in slow growth (Chen et al., 1993). Knocking out both YPK1 and YPK2 causes lethality (Chen et al., 1993). Orm2 phosphorylation in response to heat stress in the ypk1-as ypk2Δ mutant was monitored in the presence of 3-MOB-PP1, which specifically inhibits ypk1-as kinase activity. Heat stress–induced phosphorylation no longer occurs when Ypk kinase activity is abolished (Figure 4b and Supplemental Figure S4a), indicating that heat stress facilitates Orm phosphorylation through Ypk kinase activity. More important, heat-induced sphingoid base accumulation no longer occurs when Ypk kinase activity is inhibited (Figure 4c and Supplemental Figure S4b). These results not only provide strong evidence that Orm phosphorylation positively regulates sphingolipid production in vivo, but they also indicate that Ypk kinase transmits the heat stress signals to sphingoid intermediate production through phosphorylation of the Orm proteins.

Ypk1/2 are phosphorylated and activated by Pkh1/2, the homologues of mammalian PKD1 (Casamayor et al., 1999). Neither Pkh1 nor Pkh2 alone is required for cell growth, but loss of both proteins causes lethality (Casamayor et al., 1999). Consistently, heat stress–induced phosphorylation is abolished when Pkh kinase activity is inhibited (Figure 4d).

A recent study proposed that Orm2 expression levels regulate sphingolipid synthesis (Liu et al., 2012). However, as shown in Figure 3a, Orm phosphorylation starts to increase within 1 min after introduction of heat stress, reaching its maximum at around 5 min, and then decreases. The rapid time scale of these events strongly suggests that regulation of sphingolipid synthesis in response to heat is primarily due to changes in Orm phosphorylation, not to changes in Orm expression.

Together, the results shown in Figures 3 and 4 establish a physiologically relevant context for the association between sphingolipid homeostasis and Orm protein phosphorylation dynamics and identify a novel feedback pathway for temporal regulation of sphingolipid biosynthesis during the heat stress response: heat stress rapidly activates the Pkh-Ypk signaling pathway to induce Orm phosphorylation, which in turn promotes sphingolipid intermediate production. The accumulated sphingoid intermediates then trigger Orm dephosphorylation, which in turn down regulates sphingolipid biosynthesis.

Cdc55-PP2A is a key phosphatase that counteracts Ypk1 activity in Orm-mediated sphingolipid biosynthesis regulation

Orm phosphorylation state is set not only by kinases but also by phosphatases. We next sought to identify the phosphatase involved in Orm dephosphorylation. We hypothesized that the phosphatase acting on the Orm proteins is likely to be involved in...
and/or regulated by sphingolipid levels. PP2A (Figure 5a) was identified as an attractive candidate for a ceramide-activated protein phosphatase in yeast (Nicksels and Broach, 1996). Inactivation of Cdc55 or the catalytic subunits of PP2A was reported previously to suppress the endocytic defects of a mutant with impaired SPT activity (Friant et al., 2000), suggesting that PP2A may be involved in sphingolipid production. We explored the specific relationship between Cdc55-PP2A activity and Orm regulation at standard growth temperatures (25 or 30°C).

The yeast PP2A, similar to its mammalian counterparts, is a heterotrimer composed of three distinct subunits, namely A (the structural subunit, encoded by TPD3), B (the regulatory subunit, encoded by two distinct genes, CDC55 and RTS1), and C (the catalytic subunit, also encoded by two distinct genes, PP1H21 and PP1H22) (Jiang, 2006). If PP2A does dephosphorylate Orm2, lack of PP2A activity would be expected to result in increased Orm2 phosphorylation. tpd3Δ and ppk21Δ ppk22Δ showed increased in both phosphorylated (the two slowest-migrating bands) and dephosphorylated forms (the fastest-migrating band) of Orm2 (Figure 5b). The total Orm2 expression levels in these two mutants also appeared to be much higher than in wild-type cells (Figure 5b). Moreover, both of these strains have severe growth defects (Figure 5c). Thus, we conclude that it is not possible to address PP2A function, particularly in Orm phosphorylation, using these two mutants; the elevated Orm2 expression in these mutants may affect sphingolipid synthesis (Liu et al., 2012). In contrast, ppk21Δ, ppk22Δ, cdc55Δ, and rts1Δ single mutants grow relatively normally (Figure 5c). Compared to wild-type cells, Orm2 phosphorylation levels increased dramatically in cdc55Δ mutants (Figure 5b). ppk21Δ cells also showed higher Orm2 phosphorylation (Figure 5b). In contrast, the Orm2 phosphorylation patterns in rts1Δ and wild-type cells were indistinguishable (Figure 5b). These results indicate that Cdc55-PP2A activity, rather than Rts1-PP2A activity, mediates Orm dephosphorylation.

We next examined how PP2A mutants respond to the SPT inhibitor myriocin. Previous studies suggested that Orm proteins negatively regulate SPT activity and that Orm phosphorylation relieves their inhibition of SPT (Breslow et al., 2010; Figure 1). Thus, impaired PP2A activity, which increases Orm2 phosphorylation, might lead to enhanced myriocin resistance. Unfortunately, tpd3Δ and ppk21Δ ppk22Δ mutants show severe growth defects even without myriocin (Figure 5c), making assessment of their myriocin sensitivity difficult. However, ppk21Δ, ppk22Δ, and cdc55Δ single mutants showed resistance to 0.75 μg/ml myriocin, a concentration that inhibits growth of wild-type cells (Figure 5c). The same set of PP2A mutants could not survive 1 μg/ml myriocin (Figure 5c), a concentration that likely causes more complete inhibition of SPT activity. In addition, cdc55Δ rescued the growth of the lcb1-100 ts mutant at a semirestrictive temperature but not at the restrictive temperature (Figure 5d). Lcb1 is an essential component of yeast SPT (Buede et al., 1991). Thus, it appears that the myriocin resistance of the Cdc55-PP2A–deficient mutants is only observable when SPT activity is not completely blocked and thus is still regulated by Orm proteins. The rts1Δ mutant shows resistance to even 1 μg/ml myriocin (Figure 5c), implying that the rts1Δ mutant cells and cdc55Δ mutant cells are resistant to myriocin through a different mechanism.

To further explore the relationship between PP2A, the Orm proteins, and SPT regulation, we examined the myriocin sensitivity of PP2A mutants in an orm-null mutant background. We found that ppk21Δ orm1Δ orm2Δ and cdc55Δ orm1Δ orm2Δ triple mutants were not resistant to 0.75 μg/ml myriocin (Figure 6a, and b), whereas rts1Δ orm1Δ orm2Δ mutants were resistant to even 1 μg/ml myriocin (Figure 6a), establishing that Orm proteins are required for the myriocin resistance of Cdc55-PP2A–deficient mutants but not of the rts1Δ mutant.

We next tested the role of Ypk phosphorylation of the three Orm2 serines (Roelants et al., 2011) in the myriocin resistance of
Cdc55-PP2A deficient mutants. As shown in Figure 6, b and d, the pph21A orm2-3D orm1A and cdc55A orm2-3D orm1A strains show myriocin resistance, whereas the pph21A orm2-3A orm1A and cdc55A orm2-3A orm1A strains do not. These results support the conclusion that Cdc55-PP2A counteracts Ypk1 phosphorylation of the three indicated Orm2 serines in regulation of sphingolipid production. In contrast, the rts1A orm2-3A orm1A strain is resistant to myriocin (Figure 6d), which further confirms our conclusion that myriocin resistance of rts1A is independent of the Orm proteins.

A previous study demonstrated that cdc55A confers resistance to rapamycin (Jiang and Broach, 1999), which is an inhibitor of TOR signaling. Strikingly, we found that Orm proteins are required for myriocin resistance of cdc55A cells but not for their rapamycin resistance (Figure 6c). This result establishes that whereas PP2A may be involved in multiple cellular signaling pathways (Jiang and Broach, 2006), Cdc55-PP2A and Orm protein activities are linked specifically for sphingolipid biosynthesis. Furthermore, sphingoid base levels were substantially increased in cdc55A cells (Supplemental Figure S3), indicating that Cdc55-PP2A activity is involved in sphingolipid production.

PP2A activity contributes to the regulation of Orm phosphorylation dynamics in response to heat stress

We next examined the role of Cdc55-PP2A in Orm2 dephosphorylation dynamics in response to heat stress. We generated a PP2A ts mutant, pph21 E102K pph22Δ (Lin and Arndt, 1995), which allowed us to simultaneously introduce heat stress and turn off PP2A activity. As shown in Figure 7, substantially less dephosphorylated Orm2, seen as the fastest-migrating bands on a phosphate-affinity gel, appears in the PP2A ts mutant at both the 10- and 15-min time points compared with pph22Δ cells. The observation that Orm dephosphorylation was not completely abolished in the PP2A ts mutant suggests that other phosphatases may also contribute to Orm protein dephosphorylation in response to heat stress. It is also possible that PP2A activity was not completely lost in the PP2A ts mutant after the short temperature shift. Nevertheless, our results indicate that PP2A activity contributes to regulation of Orm phosphorylation dynamics in response to heat stress.

**DISCUSSION**

A model for how Orm phosphorylation regulation controls sphingolipid biosynthesis in response to stress in a kinetically coupled manner

As bioactive molecules, sphingoid intermediates transmit signals when their cellular levels change in response to various stresses (Hannun and Obeid, 2008; Dickson, 2010; Nikolova-Karakashian and Rozenova, 2010). However, both lack of sphingolipids and constitutive high levels of sphingolipids compromise cell viability (Buede et al., 1991; Chung et al., 2001). Thus, biosynthesis of sphingoid intermediates in response to stresses requires precise temporal control. Previously, very little was known about the regulatory mechanism.

Here we provided evidence supporting a model in which the conserved Phk-Ypk signaling cascade and Cdc55-PP2A facilitate/ensure rapid, transient sphingolipid production upon heat stress through regulation of Orm protein phosphorylation (Figure 8): 1) A Phk-Ypk cascade is rapidly activated upon heat stress. 2) Orm phosphorylation rapidly increases. 3) Orm phosphorylation releases inhibition of SPT activity. 4) Activated SPT promotes de novo synthesis of sphingoid intermediates. 5) Accumulated sphingoid intermediates act as signaling molecules to initiate the cellular heat shock response. 6) Data show that Orm phosphorylation rapidly increases in response to heat stress in both wild-type and Cdc55-PP2A deficient backgrounds. 7) When sphingoid intermediates are maintained constitutively, Orm2 becomes fully dephosphorylated by PP2A, which is consistent with published data (Buede et al., 1991; Chung et al., 2001).
responses, as described in the Introduction. Sphingoid intermediate accumulation also feeds back to dephosphorylate Orm proteins, possibly by inhibiting Pkh-Ypk kinase activity or/and activating Cdc55-PP2A phosphatase activity. 6) Dephosphorylated Orm proteins inhibit SPT activity.

The Pkh-Ypk cascade was previously proposed to be downstream of heat-induced sphingolipid accumulation (Dickson, 2010). However, our data indicate that the Pkh-Ypk cascade is activated in response to heat stress and induces de novo sphingolipid biosynthesis (Figure 4). Of interest, a previous in vitro study indicated that Pkh1/Pkh2 kinases are inhibited by micromolar concentrations of sphingoid bases (Friant et al., 2001). Thus, the most harmonious interpretation of our results and previous findings is that the Pkh-Ypk cascade is also regulated by heat-induced sphingolipid intermediate accumulation via a negative feedback mechanism by which increased sphingolipids inhibit Pkh-Ypk kinase-mediated phosphorylation of Orm proteins, thereby restoring SPT inhibition.

How could heat stress activate Ypk kinases to trigger Orm2 phosphorylation in such a rapid manner (Figure 3a)? Breslow et al. (2010) showed that disruption of sphingolipid biosynthesis by myriocin results in an increase in Orm phosphorylation. However, the increase in Orm phosphorylation in response to heat stress apparently is not due to low levels of sphingolipids, because both the levels of Orm phosphorylation and the levels of sphingolipid intermediates actually continue to increase at these early time points after heat stress (Figure 2C, time points between 0 and 4 min; Dickson et al., 1997; Jenkins et al., 1997; Jenkins, 2003; Wells et al., 1998; Mao et al., 1999; Skrzypek et al., 1999). Moreover, our data suggest that heat stress affects Orm phosphorylation before the myriocin-sensitive step (Figure 3e). Ypk1 activation is known to require its phosphorylation by both Pkh kinases and the TORC2 complex (Casamayor et al., 1999; Kamada et al., 2005). Recently Slm1/2 was demonstrated to play important roles in recruiting Ypk1 to the plasma membrane for activation by the TORC2
Orm phosphorylation status is highly responsive to sphingoid bases

In this study, we demonstrated that the high levels of Orm phospho-
rolation induced by heat stress decline while the sphingoid bases
(PHS) reach their peak levels (Figure 3c, time points between 5 and
15 min). Several previous studies established that sphingoid bases
and sphingoid base phosphates accumulate with similar timing
(Dickson et al., 1997; Jenkins et al., 1997). However, the heat-in-
duced ceramides peak ~10 min after the sphingoid bases reach
their peak (Dickson et al., 1997; Jenkins et al., 1997), and no obvious
changes in the levels of complex sphingolipids were observed dur-
ing 2 h of heat stress (Jenkins et al., 1997). Thus, the decrease of
Orm phosphorylation induced by heat stress is kinetically coupled
to changes in sphingoid base and sphingoid base phosphate levels
but not ceramide or complex sphingolipid levels, suggesting that

Orm phosphorylation is primarily regulated by sphingoid bases and
sphingoid base phosphates. In agreement with these conclusions,
our experiments indicate that exogenously provided sphingoid
bases are sufficient to induce Orm dephosphorylation without be-
ing converted to ceramide or to complex sphingolipids (Figure 2).

A recent study proposed that Orm1 and Orm2 may regulate
sphingolipid synthesis via two different mechanisms (Liu et al., 2012).
We observed the phosphorylation dynamics of Orm1 and Orm2
upon heat stress to be very similar (unpublished data), suggesting
that both of the Orm proteins function similarly in regulation of the
sphingolipid biosynthesis response to heat stress. However, we
found that Orm2 expression was at least 10 times higher than Orm1
expression (Supplemental Figure S1a). It is also worth mentioning
that neither C-terminally tagged Orm1 nor Orm2 is functional
(unpublished data) and that Orm1 tagged at its N-terminus with
3XFLAG cannot fulfill Orm1 function in the orm2∆ background
(Supplemental Figure S1A). Our studies indicate that Orm function
is preserved in N-terminally tagged Orm2.

Phosphate-affinity gels separated Orm2 protein into four mobil-
ity groups. Western blotting analysis revealed that the majority of
Orm proteins are moderately phosphorylated (bands in the third
group, Figure 2a) under standard growth conditions. This large pool
of moderately phosphorylated Orm protein may explain how cells
rapidly respond to various environmental stresses in a graded man-
ner. In addition, the majority of orm2-3A proteins were detected as
dephosphorylated forms, and levels of sphingoid bases were greatly
reduced in orm2-3A cells, further supporting the conclusion that
dephosphorylated Orm proteins negatively regulate sphingolipid bio-
synthesis. Given that the reduction of dephosphorylated Orm2 and
the increase in hyperphosphorylated Orm2 occur at the same time
in response to heat stress (Figure 3b, time points 2 and 5 min), SPT
activation in response to heat stress could be caused by release of
SPT inhibition by dephosphorylated Orm2, by positive regulation of
SPT by hyperphosphorylated Orm2, or both.

Cdc55-PP2A counteracts Ypk1 activity in Orm-mediated
sphingolipid biosynthesis regulation

In comparison to kinases, phosphatases often function in a less spe-
cific manner. In this study, we performed a series of experiments to
demonstrate a specific relationship between Cdc55-PP2A and the
Orm proteins in regulating sphingolipid biosynthesis. First, sphingo-
lipid biosynthesis increased in cdc55∆ mutant cells. Second, Orm proteins
were required for myriocin resistance but not for rapamycin resistance
of cdc55∆ cells. Finally, genetic analysis suggested that Cdc55-PP2A
functions to counteract Ypk1 kinase–mediated Orm phosphorylation.

In contrast, we found that Rts1, another PP2A regulatory subunit,
is involved in sphingolipid regulation in an Orm protein–indepen-
dent manner. Previous studies suggested that Sac1 binds and mod-
ulates SPT activity in a pathway independent of Orm proteins
(Breslow et al., 2010). Future investigations are needed to test
whether Rts1-PP2A is involved in Sac1 modulation of SPT activity.

PP2A was previously identified as an attractive candidate for a
ceramide-activated protein phosphatase in yeast (Nickels and
Broach, 1996), although direct evidence that ceramides (or other
sphingoid intermediates) activate PP2A has not been reported. It is
not clear whether other phosphatases are also involved in Orm phos-
phorylation. Loss of Ypk kinase activity abolished sphingolipid
biosynthesis in response to heat stress (Figure 4c), but Orm phos-
phorylation still decreased (Figure 4b, right), suggesting that Cdc55-
PP2A may affect Orm phosphorylation at least partially in a constitu-
tive manner. Because both Pkh kinases and the TORC2 complex are
required for Ypk kinase activity, an alternative explanation for our

complex (Niles et al., 2012). Of interest, both Pkh kinases and
Slm1/2 localize in punctate eisosome plasma membrane domains
(Walther et al., 2007; Grossmann et al., 2008), and the TORC2
complex localizes in small foci on the plasma membrane but not in
eisosomes (Berchtold and Walther, 2009). Another recent study
suggested that plasma membrane stress might induce relocation
of Slm1/2 and activation of TORC2 (Berchtold et al., 2012).
Thus, it is possible that heat stress may rapidly decrease the rigid-
ity of plasma membrane, resulting in release of Pkh kinase and
Slm1/2 from eisosomes, thereby triggering Ypk kinase activation.

Both the lipids and the proteins involved in this regulatory circuit
(Figure 8) are highly conserved between yeast and mammalian cells.
Thus, the mechanism we proposed here may serve a general basis
for how Orm phosphoregulation controls sphingolipid biosynthesis
in response to stress in a kinetically coupled manner.
results could be that Cdc55-PP2A controls Orm phosphorylation by negatively regulating these two upstream effectors of Ypk kinases, instead of directly dephosphorylating the Orm proteins. However, this possibility seems unlikely because Orm2 dephosphorylation should have no longer occurred upon loss of Ypk kinase activity (Figure 4b, right), which is contrary to our observation.

MATERIALS AND METHODS

Media and strain construction

The yeast strains used in this study are listed in Supplemental Table S1. They were grown in standard rich media (yeast extract/peptone/dextrose [YPD]). The primers used for cloning are listed in Supplemental Table S2.

To generate a strain expressing the 3XFLAG-fused Orm2 protein from its genetic locus, we first constructed a vector using following strategies. Two DNA fragments were obtained by PCR amplification, using primer pairs of HindIII-ORM2-F/FLAG-ORM2-interR and FLAG-ORM2-interF/Xhol-ORM2-R, respectively. The two DNA fragments were then converted into one by PCR amplification, using primer pairs of HindIII-ORM2-F/Xhol-ORM2-R. The resulting PCR product was digested and ligated into the pBluescript II KS(+) vector using the HindIII and Xhol restriction enzymes. In addition, a NAT1 (nourseothricin acetyltransferase) DNA fragment, amplified by primer pairs NatR-ORM2-F/Natr-ORM2-R, was inserted into the same vector using the HindIII and SpeI restriction enzymes. The resulting plasmid, named pBS-NAT1-FLAG-ORM2, was digested with HindIII and Xhol, and the fragment containing NAT1-FLAG-ORM2 was transformed into a yeast strain in which ORM2 had been replaced by Candida glabrata (Cg) URA3. We isolated transformants that no longer grow on a plate lacking uracil but did grow on a nourseothricin-dihydrogen sulfate (clonNAT)–containing plate.

To obtain strains expressing Ypk1-as (Ypk1 L424A), two fragments were PCR amplified from genomic DNA using primers OYS227/F and ORM2-3A-F/ORM2-3A-R, respectively. A strain expressing 3XFLAG-Orm1 was generated by a similar strategy but using different primers, listed in Supplemental Table S2.

To transform the pRS306-PPH21 E102K plasmid was integrated into the URA3 locus of a pph21Δ::CgHIS3 strain.

Lipid reagents

Phytosphingosine purified from Saccharomyces cerevisiae was obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylserine was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and stearylamine was obtained from Sigma-Aldrich (St. Louis, MO). All lipids were dissolved in methanol.

Preparation of whole-cell extracts

Yeast cells were grown to early logarithmic phase in YPD. After the indicated treatments, cold trichloroacetic acid (TCA) was added to the yeast culture to a final concentration of 20% (vol/vol). The growth medium was removed after centrifugation, and cell pellets were flash frozen in liquid nitrogen. The cells were thawed, resuspended in 5% (vol/vol) TCA, and lysed by bead beating at 4°C for 10 min. Whole-cell extracts were collected by 5 min of centrifugation at 14,000 × g at 4°C. The pellets were resuspended in SDS–PAGE sample buffer containing 50 mM dithiothreitol.

Detection of protein phosphorylation

To detect phosphorylation-dependent mobility shifts of FLAG-Orm1 and FLAG-Orm2, whole-cell extracts were loaded onto 8% SDS polyacrylamide gels containing 25 μM Phos-tag acrylamide (Wako Chemicals USA, Richmond, VA) and 25 μM MnCl2. Before transfer to nitrocellulose membranes, gels were washed twice for 10 min in 2 mM EDTA containing transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% [vol/vol] methanol) and then once for 10 min in transfer buffer without EDTA. Membranes were then probed with 1:4000 mouse anti-FLAG (Sigma-Aldrich).

Lipid analysis by high-performance liquid chromatography

Extraction and processing of sphingoid bases from yeast cells for fluorescence high-performance liquid chromatography (HPLC) analysis using the AQC reagent (Waters, Milford, MA) was performed as described previously (Lester and Dickson, 2001). Sampling of heat-stressed cells was as described. HPLC analysis was performed using a C18 column (4.6 × 250 mm, XDB-C18; Hewlett-Packard, Palo, Alto, CA) on a Shimadzu LC10A series liquid chromatography system. Isocratic elution was carried out for 60 min at a flow rate of 1.0 ml/min. Lipid-reacted AQC reagent was excited with 244-nm UV radiation, and the resultant emission signal at 398 nm was detected. C18-PHS reacted with the AQC reagent was used as a standard for quantification.

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