Sphingolipids Signal Heat Stress-induced Ubiquitin-dependent Proteolysis*

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Sphingolipids are essential eukaryotic membrane lipids that are structurally and metabolically conserved through evolution. Sphingolipids have also been proposed to regulate eukaryotic stress responses as novel second messengers. Here we show that, in Saccharomyces cerevisiae, phytosphingosine, a putative sphingolipid second messenger, mediates heat stress signaling and activates ubiquitin-dependent proteolysis via the endocytosis vacuolar degradation and 26 S proteasome pathways. Inactivation of serine palmitoyltransferase, a key enzyme in generating endogenous phytosphingosine, prevents proteolysis during heat stress. Addition of phytosphingosine bypasses the requirement for serine palmitoyltransferase and restores proteolysis. Phytosphingosine-induced proteolysis requires multiubiquitin chain formation through the stress-responsive lysine 63 residue of ubiquitin. We propose that heat stress increases phytosphingosine and activates ubiquitin-dependent proteolysis.

*Sphingolipids are complex lipids containing a sphingoid base, which is a long chain amino base. Sphingolipids comprise indispensable structural components of all known eukaryotic plasma membranes and also regulate signal transduction elements including protein kinase C (1). Sphingosine, ceramide, sphingosine 1-phosphate, and other sphingolipid derivatives are also known to play central roles in apoptosis, cellular senescence, cell cycle regulation, inflammation, tumor development, and intracellular calcium mobilization (2–4).

Despite the growing number of cellular functions regulated by sphingolipids and their derivatives, the molecular mechanisms by which these regulatory functions are executed in mammalian cells are poorly understood (5–7). Being ubiquitous and essential components of eukaryotic plasma membranes, sphingolipids are evolutionarily conserved from yeast to humans (8, 9). The yeast Saccharomyces cerevisiae has several advantages as a model system to study sphingolipid-mediated cellular regulation. First, the basic structure and metabolism of sphingolipids are conserved between yeast and mammals, and yet yeast has only three major species of sphingolipids, whereas mammalian cells may contain more than 300 distinct molecular species (10). Second, yeast is a genetically tractable organism whose genome has been completely sequenced (11). Lastly, many yeast genes encoding sphingolipid biosynthetic and metabolic enzymes have been recently identified (12). The controlled expression of these genes can be exploited to modulate intracellular levels of certain sphingolipids and their derivatives.

The LCB1 and LCB2 genes encode serine palmitoyltransferase, which catalyzes the first committed step in yeast sphingolipid biosynthesis, the condensation of L-serine and palmitoyl-CoA to produce 3-ketodihydrosphingosine (KDS) (13, 14). Recently, heat stress was shown to increase cellular levels of sphingoid bases (dihydrosphingosine (DHS) and phytosphingosine (PHS)) and ceramides with little effect on the levels of complex sphingolipids (15, 16). This increase in sphingoid bases is blocked in an lcb1 mutant, suggesting the de novo synthesis of sphingoid bases by serine palmitoyltransferase.

This suggests potential involvement of sphingolipids in stress response, but the biological consequences of such an increase in the levels of cellular sphingoid bases and ceramides have been obscure. Moreover, these studies could not differentiate whether sphingolipids maintain plasma membrane integrity or are involved in relaying stress signals to downstream effectors. In this study, we set out to understand the role of sphingolipids in yeast stress response and the mechanism by which these lipids exert biological effects. This study led us to identify an essential function for serine palmitoyl transferase (SPT) and the de novo synthesis of PHS in mediating stress effects on nutrient permeases via a mechanism involving ubiquitin-dependent proteolysis.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—S. cerevisiae strains used in this study include the following: JK9–3da (MATa trp1 his3 leu2–3, 112 ura3–52 rme1 HMLa) (17) and isogenic mutant strains NC86 (DOA4Δ::URA3) (18), NC23 (DOA4Δ::G418), and NC227 (UFL4::G418); RH406–2A (MATa leu2 trp1 ura3 3α) and isogenic lcb1 temperature-sensitive strain RH614–2C (lcb1Δ–100) (19); and isogenic lcb1 temperature-sensitive strain 27061 (UFL4::URA3 [Ub K63R] [pUB100]) (20, 21). Plasmids used here are as follows: YEp352DF (2 μ MCA4 UR4) (22), Deg1–βGal (CEN URA3 Deg1–βGal) (23), and pTod2 (2 μ MCA4 wt

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Stock solutions were made in ethanol at 20 mM, stored at Charleston, SC). The quality of these sphingolipid derivatives was gifts from Dr. Alicja Bielawska (Medical University of South Carolina, and C2-phytoceramide (PHC), which was (STA) were purchased from Sigma, DHS was from Biomol, and KDS medicinals, Inc.) for 10 min at room temperature.

Yeast transformation followed the protocol developed by Gietz et al. (27). Gene disruptions were carried out as described previously (28). In short, open reading frames were replaced by the PCR products consisting of the G418<sup>+</sup> gene cassette flanked by 43-base pair sequence homology to targeted genes on both sides of an open reading frame. Each gene disruption was confirmed by PCR, which was designed to amplify the specific chimeric junction of the target gene and the G418<sup>+</sup> cassette.

Preparation of Sphingolipid Derivatives—PHS and stearylamine (STA) were purchased from Sigma, DHS was from Biomol, and KDS and C<sub>2</sub>-phyto酰aramide (PHC), which was N-acetylated from PHS, were gifts from Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). The quality of these sphingolipid derivatives was controlled by thin-layer chromatography for apparent homogeneity. Stock solutions were made in ethanol at 20 mM, stored at −20 °C, and warmed up before use to redissolve precipitates. Tritiated H<sub>2</sub>-histidine, L-leucine, L-tryptophan, L-serine, and uracil (1 µg/Cm/µl each) were purchased from American Radiolabeled Chemicals Inc.

Yeast Growth Assays—Measurement of yeast growth was carried out as described previously (29). Briefly, in liquid culture, an overnight culture of cells at exponential growth phase was diluted into fresh medium containing indicated lipids or ethanol as a control. While incubating in a shaking incubator at 30 °C, growth was monitored at a given time by measuring absorbance at 600 nm (A<sub>600</sub>), and the numbers were converted into cell density (cells/ml), using a pre-configured conversion table, when it was necessary. On solid medium, a small amount of cells from a single colony were streaked by three successive uses of toothpicks, or exponential-phase cells in liquid culture were plated onto medium. Plates were incubated at 30 °C for 2 days and photographed for our record.

Sphingolipid Analysis—Isogenic wild-type and lcb1–100 mutant cells were grown at 24 °C until exponential phase, harvested, and resuspended in media at either 24 or 39 °C. After 15 min, an equal number of cells were harvested, processed for high performance liquid chromatographic analysis, and normalized by a phosphate assay as described previously (15).

Northern Analysis—Log-phase cells were treated with the indicated sphingolipids in the presence of ethanol for 2 h, and total RNA was prepared using the RNeasy Mini Kit (QIAGEN). 20 µg of total RNA per lane were loaded onto 1% agarose-formaldehyde gel, and transfer and hybridization were carried out as described previously (30). Probes were prepared with a Random Primed DNA Labeling Kit (Roche Molecular Biochemicals). Radioactive bands were visualized by autoradiography.

RESULTS AND DISCUSSION

In this study we investigated whether sphingolipids mediate stress signals in <i>S. cerevisiae</i>. We have found that PHS specifically inhibits import of various nutrients, such as uracil, into yeast cells. Here we determined the mechanisms by which PHS inhibits uracil transport. Uracil import is mediated by the uracil permease (Fur4), a member of the major facilitator superfamily (36). Fur4 activity is known to be down-regulated upon heat stress and starvation (22, 37), suggesting Fur4 is a target of stress response pathways. We tested whether the inhibitory effect of PHS on uracil permease activity is due to a decrease in levels of the Fur4 protein. When yeast cells were exposed to PHS (20 µM), Fur4 protein levels started to decrease by 15 min and were almost undetectable by 2 h (Fig. 1A). In contrast, PHS did not alter the level of a control protein (Cpr1) (Fig. 1A). In contrast to PHS, other closely related lipids including DHS, KDS, C<sub>2</sub>-PHC, and STA had little or no effect on Fur4(Fig. 1B).

Next, we determined whether the effect of PHS on Fur4 activity was physiologically relevant. Previous studies have demonstrated that PHS levels increase upon heat stress (15, 16) and that heat stress inhibits Fur4 activity (37). We tested whether PHS is necessary for Fur4 degradation in response to heat stress. To address this, we utilized an <i>S. cerevisiae</i> strain containing a conditional mutation in the <b>LCB1</b> gene (lcb1–100), which encodes SPT, an essential enzyme that catalyzes the first and rate-limiting step in de novo synthesis of PHS (13). The lcb1–100 strain is a temperature-sensitive (ts) mutant strain, whose growth is arrested at 37 °C and slow even at the permissive temperature (24 °C) (18). This suggests that SPT activity in the lcb1–100 strain is impaired to some degree even at 24 °C. The ts phenotype is reversible if the incubation temperature is returned from 37 to 24 °C or if the growth medium is supplemented with 5 mM PHS when the mutant is grown at 37 °C, indicating that the growth defects of the lcb1–100 strain are due to a defect in sphingolipid synthesis. Because the lcb1–100 allele was originally isolated as an endocytosis mutant (end8–1) and later shown to be a temperature-sensitive allele of the <b>LCB1</b> gene, we first tested whether this mutation affects SPT activity and PHS generation. In wild-type cells 15 min of heat stress increased cellular levels of PHS severalfold. In contrast, in the lcb1–100 mutant cells, heat stress had little effect on PHS levels (Fig. 2A). We next evaluated Fur4 degradation in this model. With a short time lag in wild-type cells, Fur4 degradation followed the rise in PHS levels such that upon heat stress, Fur4 levels dropped soon after the increase in PHS and completely disappeared by 30 min of heat stress (Fig. 2B). In contrast, in the lcb1–100 strain upon heat stress, which

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<sup>2</sup> N. Chung, Y. A. Hannun, and L. M. Obeid, unpublished data.
blocked the generation of PHS by inactivating SPT, the Fur4 protein was stable over a prolonged incubation. These findings suggest that production of PHS by SPT is required for Fur4 degradation. To establish that the function of Lcb1 is to supply PHS, we tested whether the addition of exogenous PHS to lcb1–100 mutant cells would restore Fur4 degradation. SPT was first inactivated at 37 °C for 30 min to deplete endogenous PHS, and exogenous PHS was then added under conditions non-permissive for SPT activity (37 °C). As shown in Fig. 2C, exogenous PHS restored Fur4 degradation in lcb1–100 mutant cells, albeit not to the level of that in LCB1 wild-type cells.

Next, we investigated the mechanism by which PHS reduces Fur4 protein. By Northern blot analysis, PHS does not repress transcription of the FUR4 gene (Fig. 3A). Nutrient permeases, including the uracil permease, are known to undergo proteolytic degradation (38, 39). The uracil permease can be ubiquitinated in its cytoplasmic tail, which signals endocytosis to the vacuole and degradation (37). The ubiquitin ligase, Npi1, and the deubiquitinase, Doa4, are required for Fur4 endocytosis and degradation (22, 40). In both npi1 and doa4 mutant yeast strains, the Fur4 protein was no longer degraded upon addition of PHS (Fig. 3B). Moreover, the npi1 and doa4 mutant strains were also PHS-resistant. Thus, Npi1 and Doa4 function downstream of PHS in the pathway regulating Fur4 stability.

We next addressed whether PHS activates ubiquitination of Fur4 or regulates a step in Fur4 endocytosis independent of the ubiquitination. To resolve this issue, we analyzed the effects of PHS on the Deg1–βGal protein. Deg1–βGal is a fusion protein between the amino terminus of the 26 S proteasome subunit (23). This fusion protein is ubiquitinated and is subject to degradation by the 26 S proteasome pathway, which is independent of endocytosis (38, 39). As shown in Fig. 4, PHS (20 μM) induced degradation of Deg1–βGal. To confirm that PHS-induced degradation of Deg1–βGal was via the proteasome pathway, we analyzed the fate of the Deg1–βGal protein in a doa1 mutant strain. DOA1 encodes a regulatory component of the proteasome pathway and is required for degradation of the α2 repressor (23). As shown in Fig. 4, the Deg1–βGal protein was not degraded when doa1 mutant cells were exposed to PHS. Thus, PHS also stimulates protein degradation through the proteasome pathway, supporting the hypothesis that PHS regulates ubiquitination.

We addressed how PHS regulates ubiquitination and degradation of Fur4 and Deg1–βGal. Diverse types of stress are known to increase the intracellular ubiquitin pool via transcriptional activation of the polyubiquitin gene UBI4 (35). PHS induced UBI4 transcription; however, by Western blot analysis, the levels of ubiquitin were largely unchanged by PHS (data not shown), and Fur4 and Deg1–βGal were still degraded upon addition of PHS in ubi4Δ mutant cells at a rate similar to UBI4 wild-type cells (data not shown). We then examined other aspects of the ubiquitin system that may have a role in PHS-induced proteolysis. Ubiquitination involves linkage of the carboxyl-terminal glycine residue of ubiquitin to the ε-amino group of a lysine residue in a target protein. Monoubiquitinated proteins can be further ubiquitinated through any of three lysine residues on the first ubiquitin; Lys-29, Lys-48, or Lys-63 (24). Polyubiquitination serves to increase the efficiency and rate of proteolysis (40). We tested whether PHS increases polyubiquitination by measuring the formation of ubiquitin-ub (ub-ub) dimers from monomeric ubiquitin in the presence of PHS. As shown in Fig. 5A, PHS increased ub-ub dimer formation in a dose-dependent manner. We next measured PHS-induced ub-ub dimer formation through specific lysine residues by utilizing ubiquitin mutants in which two of three lysines were mutated to arginines. Interestingly, PHS increased polyubiquitination at all three lysine residues (Fig. 5B). Among these lysine residues, Lys-63 is suggested to be involved in stress responses (24) and polyubiquitination of the Fur4 permease (40), making it a likely candidate target for PHS. To determine whether polyubiquitination through Lys-63 is required for PHS-induced proteolysis, we used mutant yeast strains that express either wild-type ubiquitin or a Lys-63-Arg mutant (K63R) as the sole source of ubiquitin. PHS-induced
proteolysis of both Fur4 and Deg1-βGal was severely impaired in K63R ubiquitin mutant cells compared with wild-type cells (Fig. 5C). Because K63R ubiquitin, like wild-type ubiquitin, can be used in a monoubiquitination reaction, this demonstrates that polyubiquitination through Lys-63 is critical for the PHS-induced proteolysis. We conclude that PHS-induced degradation of Fur4 and Deg1-βGal is executed by a PHS-stimulated polyubiquitination activity that extends through the stress-responsive Lys-63 residue of ubiquitin.

Sphingolipids have been implicated in stress responses, but it has been unclear whether these lipids play an essential structural role or function as signal transducers. In this report we demonstrate an essential role for sphingolipids in signaling during heat stress response by showing that PHS targets both the Fur4 protein and the α2 repressor for degradation through two different ubiquitin-mediated proteolysis pathways. We propose a model in which heat stress activates PHS synthesis by SPT, which in turn signals to activate the ubiquitin system. Considering the ancient origin and evolutionary conservation of both sphingolipids and the ubiquitin system, this stress-sphingolipid-ubiquitin mechanism is likely not limited to yeast cells.

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REFERENCES

1. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J. Biol. Chem. 261, 12624–12630
2. Hannun, Y. A. (1997) in Sphingolipid-mediated Signal Transduction (Hannun, Y. A., ed) R. G. Landes Company and Chapman & Hall, Austin, TX
3. Merrill, A. H., Jr., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayan, J. A., Schroeder, J. J., Riley, R. T., Voss, K. A., and Wang, E. (1997) Tissue Appl. Pharmacol. 142, 229–225
4. Spiegel, S., and Merrill, A. H., Jr. (1996) FASEB J. 10, 1388–1397
5. Hannun, Y. A. (1996) Science 274, 1855–1859
6. Spiegel, S., Foster, D., and Kolesnick, R. (1996) Cell 85, 159–167
7. Kolesnick, R. N., and Kronke, M. (1998) Annu. Rev. Physiol. 60, 643–665
8. Hakomori, S. (1981) in Handbook of Lipid Research: Sphingolipid Biochemistry (Kanfer, J. N., and Hakomori, S., eds) Vol. 3, pp. 1–150; Plenum Publishing Corp., New York
9. Hannun, Y. A., and Bell, R. M. (1989) Science 243, 500–507
10. Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
11. Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Philippsen, P.,推进m H., and Oliver, S. G. (1996) Science 274, 546–547
12. Dickson, R. C. (1998) Annu. Rev. Biochem. 67, 27–48
13. Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L., and Dickson, R. C. (1991) J. Bacteriol. 173, 4325–4332
14. Nagiec, M. M., Baltisberger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7899–7902
15. Jenkins, G. M., Richards, A., Wahl, T., Mao, C., Obeid, L., and Hannun, Y. (1997) J. Biol. Chem. 272, 32569–32572
16. Dickson, R. C., Nagiec, E. E., Krzyzypke, M., Tillman, P., Wells, G. B., and Lester, R. L. (1997) J. Biol. Chem. 272, 30196–30200
17. Heitman, J., Movva, N. R., Hiestand, P. C., and Hall, M. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1948–1952
18. Sutterlin, C., Diering, T. L., Schümmoller, P., Schroder, S., and Riezman, H. (1997) J. Cell Sci. 110, 2703–2714
19. Heim, C., Springael, J. Y., Volland, C., Haguenauer-Tsapis, R., and André, B. (1995) Mol. Microbiol. 18, 77–87
20. Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Croteau, S. T., and Obeid, L. M. (1994) Mol. Cell. Biol. 14, 5501–5509
21. Spence, J., Sadis, S., Hamza, A. L., and Finley, D. (1995) Mol. Cell. Biol. 15, 1265–1273
22. Galan, J. M., Moreau, V., André, R., Volland, C., and Haguenauer-Tsapis, R. (1996) J. Biol. Chem. 271, 10946–10952
23. Hochstrasser, M., and Varshavsky, A. (1996) Cell 85, 697–708
24. Arnason, T., and Elliston, M. J. J. (1994) Mol. Cell. Biol. 14, 7876–7883
25. Galan, J. M., and Finke, G. R. (1999) Methods in Enzymology Guide to Yeast Genetics and Molecular Biology (Guthrie, C., and Finke, G. R., eds) Vol. 194, Academic Press, San Diego, CA
26. Wells, G. B., and Lester, R. L. (1983) J. Biol. Chem. 258, 10290–10293
27. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Mol. Cell. Biol. 12, 453–464
28. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) Yeast 10, 1793–1808
29. Chung, N., and Obeid, L. M. (2000) Methods Enzymol. 311, 319–331
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY
31. Romanos, M. A., Scorrer, C. A., and Clare, J. J. (1995) in DNA Cloning: A Practical Approach (Glover, D. M., and Hames, B. D., eds) 2nd Ed., pp. 123–167, Oxford University Press, Oxford
32. Harlow, E., and Lane, D. (1998) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
33. Galan, J. M., Cantele, B. N., Volland, C., Nambo, O., and Haguenauer-Tsapis, R. (1998) FASEB J. 12, 315–323
34. Cardenas, M. E., Lim, E., and Heitman, J. (1995) J. Biol. Chem. 270, 21097–21002
35. Finley, D., Okunayak, E., and Varshavsky, A. (1987) Cell 48, 1035–1046
36. Chevallier, M. R. (1982) Mol. Cell. Biol. 2, 977–984
37. Volland, C., Urban-Grimal, D., Geraud, G., and Haguenauer-Tsapis, R. (1994) Mol. Cell. Biol. 14, 5501–5509
38. Volland, C., Moreau, V., André, B., Volland, C., and Haguenauer-Tsapis, R. (1996) J. Biol. Chem. 271, 10946–10952
39. Hochstrasser, M., and Varshavsky, A. (1996) Cell 84, 813–816
40. Herskoe, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
41. Galan, J., and Haguenauer-Tsapis, R. (1997) EMBO J. 16, 5847–5854