A ceramide-activated protein phosphatase mediates ceramide-induced G₁ arrest of *Saccharomyces cerevisiae*

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Certain mammalian growth modulators, such as tumor necrosis factor α , interleukin-1 β , and γ -interferon, induce an antiproliferative response—terminal differentiation, apoptosis, or cell cycle arrest—through a novel signal transduction pathway mediated by the lipid ceramide as a second messenger. Both a ceramide-activated protein phosphatase and a ceramide-activated protein kinase have been implicated in transmitting the signals elicited by ceramide. We have determined that ceramide addition to the yeast *Saccharomyces* causes a similar antiproliferative response, resulting in arrest of cells in the G₁ phase of the cell cycle. We have also determined that yeast cells contain a ceramide-activated protein phosphatase composed of regulatory subunits encoded by *TPD3* and *CDC55* and a catalytic subunit encoded by *SIT4*. Because mutation of any one of these three genes renders strains resistant to ceramide inhibition, we conclude that the G₁ effects of ceramide are mediated at least in part by the yeast ceramide-activated protein phosphatase. These results highlight the conservation of signaling systems in yeast and mammalian cells and provide a novel approach to dissecting this ubiquitous signal transduction pathway.

[Key Words: *Saccharomyces cerevisiae*; ceramide; protein phosphatase; G₁ arrest; signal transduction pathway]

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The biological activities of a number of mammalian growth modulators—including tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β), γ -interferon, and vitamin D₃—are mediated at least in part by a novel signal transduction pathway using the lipid ceramide as a second messenger (Kolesnick 1992; Hannun 1994; for review, see Hannun and Bell 1993; Kolesnick and Golde 1994). Ceramide production and accumulation are initiated by agonist-induced hydrolysis of plasma membrane sphingomyelin to ceramide by a sphingomyelinase, resulting in activation of both a plasma membrane-bound protein kinase and a cytoplasmic protein phosphatase (Dobrowsky and Hannun 1992; Heller and Kronke 1994; Liu et al. 1994). The biological response of the target cell to activation of the ceramide signaling cascade varies depending on cell type, but the spectrum of responses include Rb-mediated cell cycle arrest, induction of apoptosis, and initiation of terminal differentiation—all processes involving regulatory decisions during the G₁ phase of the cell cycle (Hannun and Bell 1993; Obeid et al. 1993; Jarvis et al. 1994; Dbaido et al. 1995; Jayadev et al. 1995).

Identification of ceramide as a second messenger in mammalian cells rests on several observations. First, ceramide rapidly accumulates in sensitive cell lines in re-

sponse to the appropriate agonist application, with lipid accumulation preceding any biological responses of the cell-to-ligand stimulation (Okazaki et al. 1990; Obeid et al. 1993). Accumulation is dependent on the presence of appropriate receptors, and agonist-induced ceramide accumulation can be reconstituted in cell-free membrane preparations (Dressler et al. 1992; Yanaga and Watson 1992). Second, cell-permeable ceramide analogs or exogenously applied bacterial sphingomyelinases can induce many of the ligand-dependent cellular responses (Mathias et al. 1993; Dressler et al. 1992). For instance, ceramide induces the same apoptotic effects on certain monocytic and fibrosarcoma cells as does treatment with TNF α (Obeid et al. 1993; Jarvis et al. 1994). Third, treatment of sensitive cells with other phospholipases or addition of other lipids, including stereoisomers of ceramide, do not mimic ligand-induced effects (Bielawska et al. 1992a, 1993). These results underscore the specificity of ceramide in promoting the appropriate biological response. Thus, substantial evidence supports the hypothesis that ceramide serves as a second messenger mediating many of the biological effects of several growth modulators.

The immediate targets of ceramide in its role as second messenger are a ceramide-activated protein kinase (CAPK) and a ceramide-activated protein phosphatase (CAPP). Kolesnick and his colleagues have identified a

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membrane-bound protein kinase from mammalian cells that is activated 5- to 10-fold by addition of cell-permeable ceramide analogs (Liu et al. 1994). This Ser/Thr protein kinase phosphorylates endogenous epidermal growth factor (EGF) receptor in response to addition of ceramide to A431 cells and shows enhanced activity immediately following treatment of HL-60 cells with TNF α (Kolesnick 1991). The 97-kD CAPK is a member of the proline-directed protein kinase family that phosphorylates the serine or threonine residue in an X-Ser/Thr-Pro-X context (Liu et al. 1994). However, the natural substrates for CAPK have not been identified, and, accordingly, its role in ceramide-mediated signal transduction remains unresolved.

A second potential primary mediator of the ceramide signaling pathway is CAPP. Hannun and his colleagues have shown that increased ceramide levels stimulate a cytosolic protein phosphatase activity in HL-60 cells (Okazaki et al. 1990), monoblastic leukemia cells (Bielawska, et al. 1992b), and rat glioblastoma cells (Obeid et al. 1993). Cation requirements and okadaic acid sensitivity indicate that the CAPP is a member of the 2A family of protein phosphatases (PP2A), all of which are heterotrimeric proteins comprised of a small catalytic subunit, C, and two larger regulatory subunits, A and B (Cohen 1989; Mumby and Walter 1993). The fact that the biological responses of cells to ceramide treatment, such as down-regulation of *c-myc* expression or apoptosis, are blocked by okadaic acid, a specific inhibitor of protein phosphatases in the 2A family, argues that CAPP plays a required role in ceramide-induced signaling (Hannun et al. 1993). Several PP2A species exist in the cell, and although CAPP has yet to be identified at the molecular level, evidence suggests that CAPP is distinct from the major PP2A species (Dobrowsky et al. 1993). Thus, although CAPP is a prime candidate for mediating ceramide signaling in mammalian cells, its identity and its connection to the downstream targets of ceramide activation remain elusive.

Like mammalian cells, the yeast *Saccharomyces* contains sphingolipids, which are synthesized from ceramide by a pathway essentially identical to that in mammalian cells (Lester and Dickson 1993). The composition of yeast sphingolipids is much less complex than that of mammalian cells, consisting of only three types. Sphingolipids are found exclusively in the plasma membranes of yeast and are essential for cell viability (Wells and Lester 1983; Patton and Lester 1991). Mutants that are unable to synthesize phytosphingosine or dihydrosphingosine are inviable unless supplemented with these lipids. Thus, sphingolipids play a critical but as yet undefined role in yeast physiology.

Insight into a possible role for ceramide in yeast has emerged from recent work from Fishbein et al. (1993), who demonstrated that micromolar quantities of a cell-permeable derivative of ceramide [C_2 -ceramide] caused a dose-dependent inhibition of growth of wild-type strains of *Saccharomyces*. These investigators showed that extracts of *Saccharomyces* contained a ceramide-activated Ser/Thr phosphatase activity. Because this activity

could be inhibited by okadaic acid, this yeast CAPP, like mammalian CAPP, is a member of the 2A class of protein phosphatases. The structural and stereospecific requirements for activation of the phosphatase matched those for inhibition of cell growth, suggesting that yeast CAPP might in some way mediate the inhibitor effects of ceramide on cell growth.

We have extended these observations, as described in this paper, by demonstrating that ceramide inhibits G_1 progression in yeast. In addition, we have identified the components of the yeast CAPP and demonstrated that strains carrying mutations in any of these genes are resistant to the action of ceramide. Finally, additional genetic analysis suggests that ceramide exerts its antiproliferative effects at least in part by antagonism of the cAMP-dependent protein kinase, the most downstream component of the RAS pathway in yeast. These results document the existence of a ceramide-mediated signaling system that modulates cell growth of yeast, highlight the conservation of signaling mechanisms in eukaryotic cells, and offer an alternative approach to dissecting this novel signaling pathway.

Results

Yeast CAPP is a PP2A species

As a means of developing a model system for evaluating ceramide-mediating signaling in eukaryotes, we have examined the nature of CAPP in yeast. As reported previously (Fishbein et al. 1993) and as shown in Figure 1, all yeast strains examined contain a cytosolic protein phosphatase that can be activated by C_2 -ceramide (acetyl ceramide, a relatively soluble ceramide derivative), with a half-maximal activation of $\sim 4 \mu\text{M}$. The ceramide-stimulated activity was cation independent and sensitive to

Figure 1. C_2 -ceramide induces a dose-dependent CAPP activation in yeast cytosolic extracts. Cytosol (125 μg) from strains YCM35 (●), S288C (○), and W303 (▲) was incubated in the presence and absence of the indicated concentrations of C_2 -ceramide. CAPP activity was assayed as described in Materials and methods.

nanomolar concentrations of okadaic acid (data not shown), confirming the prior report that yeast CAPP is a member of the protein phosphatase 2A family.

We fractionated the cytosolic CAPP activity from strain YCM35 over several chromatographic columns. As shown in Figure 2, yeast cytosol resolved by DE52 chromatography yielded three peaks of PP2A activity, with CAPP activity copurifying with the major peak of

Figure 2. Elution profiles of PP2A and CAPP activity. (A) DEAE-Sephacel chromatographic profile of yeast cytosolic PP2A and CAPP activity from strain YCM35. (B) Mono-Q chromatographic profile of DEAE-Sephacel peak 2 PP2A and CAPP activity. (C) Polylysine-agarose chromatographic profile of Mono-Q peak 1 PP2A and CAPP activity. Fractions (4 ml) were collected and assayed in the absence of C_2 -ceramide for PP2A activity (●) and in the presence of $10 \mu M$ C_2 -ceramide for CAPP activity (○) as described in Materials and methods. NaCl gradient profiles are indicated by the solid lines. The CAPP activity in the final fraction from the polylysine column was enriched 35-fold over the initial cytosolic extract with a yield of 13%. Neither CAPP activity nor Sit4p or Tpd3p was detected in the flowthrough fractions of any of the columns.

PP2A activity. The minor fractions of phosphatase activity may represent heterodimeric and homodimeric forms of PP2A that are not responsive to ceramide (see below). Further fractionation of the major PP2A activity from the DE52 column (peak 2) by Mono-Q ion exchange chromatography followed by polylysine-agarose chromatography indicated that CAPP copurified with the major PP2A species through extensive purification. The copurification of CAPP and PP2A over several chromatographic steps supports the conclusion that as in mammalian cells, yeast CAPP is a PP2A species.

Tpd3p and Cdc55p are regulatory subunits of a yeast CAPP

As a first step in identifying the components of yeast CAPP, we examined whether known PP2A components copurify with CAPP during purification. In a screen designed to identify mutants of yeast that were defective in biosynthesis of tRNAs, previously we identified a gene, *TPD3*, that is required for efficient transcription of a variety of tRNA genes (van Zyl et al. 1989) and that encodes a protein homologous to the A regulatory subunit of mammalian type 2A protein phosphatase (van Zyl et al. 1992). We had demonstrated further that the product of the *TPD3* gene, Tpd3p, is a component of a yeast type 2A protein phosphatase, in that antibodies raised against Tpd3p could precipitate significant phosphatase activity from crude extracts and the precipitated activity exhibited substrate specificity, cation requirements, and okadaic acid sensitivity equivalent to those of a 2A phosphatase. By the Western blot analysis of fractions from the chromatographic fractionations shown in Figure 2, we find that Tpd3p copurifies with CAPP and the major 2A species (Fig. 3). These results are consistent with an assignment of Tpd3p as an A regulatory subunit of CAPP.

Further analysis indicates that, although Tpd3p is a component of CAPP, CAPP is not the major PP2A species in the cell. Using antibodies raised against Tpd3p, we find that we can immunodeplete ~85% of the CAPP but only 20% of the total phosphatase 2A in the cell (Fig. 4). This latter value is consistent with our previous measurements (van Zyl et al. 1992). These results indicate that Tpd3p is a component of most, if not all, of the CAPP in the cell and that unstimulated CAPP is a minor fraction of the total PP2A activity of the cell.

A second potential component of CAPP is the product of the *CDC55* gene, which encodes a protein with significant homology to the B subunit of mammalian type 2A protein phosphatases (Healy et al. 1991). *tpd3* strains and *cdc55* strains exhibit the exact same morphological phenotype at reduced temperature, suggesting that Tpd3p and Cdc55p are both components of a common PP2A species (van Zyl et al. 1992). To confirm the role of Tpd3p and assess the role of Cdc55p in CAPP function, we measured CAPP activity in various mutant strains of yeast. Consistent with the hypothesis that Tpd3p is a component of CAPP, strains carrying a *tpd3Δ* allele—that is, that contain no Tpd3p protein—have normal levels of

Figure 3. Immunological identification of Tpd3p and Sit4p in CAPP chromatographic fractions. Fractions (pooled in groups of three) from each chromatographic step shown in Fig. 2 were resolved by SDS-polyacrylamide gel electrophoresis under denaturing conditions on 10% slab gels. Proteins were transferred to nitrocellulose and probed with a mixture of purified anti-Tpd3p IgG antibodies that were raised against a *trpE*-*TPD3* fusion protein (van Zyl et al. 1992) and Sit4p antisera. The positions of migration of Sit4p and Tpd3p were determined from fractionation of extracts from appropriate wild type and deletion strains (Sutton et al. 1991; van Zyl et al. 1992; data not shown). (A) Sepharose fractions; (B) Mono-Q fractions; (C) polylysine-agarose fractions.

unstimulated PP2A activity but completely lack CAPP activity (Fig. 5). Restoration of the wild-type *TPD3* gene to this strain by introducing it on a centromeric plasmid completely restores CAPP activity. Similarly, we find that strains lacking Cdc55p (*cdc55Δ*) also completely lack CAPP activity (Fig. 5). Again, CAPP activity can be fully restored by reintroducing *CDC55* on a centromeric

Figure 4. Immunoprecipitation of PP2A and CAPP activity using IgG anti-Tpd3p antibodies. Cytosolic extracts (25 μl) from strain YCM35 were incubated in the presence of the indicated concentrations of anti-Tpd3p IgG antibodies. Antibodies were precipitated using protein A-sepharose. Supernatants were subsequently assayed for remaining PP2A activity (●) and CAPP activity (○). Similar immunoprecipitation of the extract using preimmune serum did not result in any loss of CAPP activity.

plasmid. We note that strains containing point mutations in *tpd3* or *cdc55* that yield temperature-conditional phenotypes contain substantial levels of CAPP activity when grown at the permissive temperature. In conjunction with the immunoprecipitation experiments described above, these data indicate that Cdc55p and Tpd3p comprise the A and B subunits of yeast CAPP.

Figure 5. Cytosolic CAPP activity from various mutant strains. Cytosolic extracts (125 μg) from the indicated yeast strains were assayed for PP2A activity (shaded bars) and CAPP activity (solid bars) as described in Materials and Methods using 5 μM [³²P]phosphohistone as a substrate. The results are the average values of five separate experiments. In all cases, individual measurements differed from the average by <5%. Specific gene mutations are indicated. Refer to Table 1 for strain genotype.

This interpretation is consistent with the genetic observation described above that *tpd3* and *cdc55* mutants have the same phenotypes and, as described below, have the same biological response to exogenously applied ceramide.

Sit4p is the primary catalytic subunit of yeast CAPP

We have used the same assay to identify the catalytic subunit of CAPP in yeast. To date, five yeast genes that are structurally related to type 2A protein phosphatase catalytic subunits have been identified in *Saccharomyces*. *PPH21* and *PPH22* are highly homologous genes that encode the major PP2A catalytic subunit in the cell (Sneddon et al. 1990). Deletion of either gene eliminates approximately half of the PP2A activity in the cell, and strains lacking both genes lack >90% of the activity. Such *pph21 pph22* strains are viable but grow very poorly. *SIT4* encodes a protein with homology to the catalytic subunits of mammalian type 1 and type 2A protein phosphatases (Arndt et al. 1989). Deletion of *SIT4* in strains lacking the *SSD1* gene is lethal, and temperature-conditional alleles of *SIT4* in this background arrest in G₁ when shifted to the nonpermissive temperature (Sutton et al. 1991). In an *SSD1* background *sit4* strains are viable, although they grow slowly and have a larger than normal abundance of unbudded cells (the function of *SSD1* is unknown, but, in addition to its effects on *sit4* mutations, its deletion exacerbates the phenotypes of mutants in the Ras/cAMP pathway and alters the phenotype of certain *cdc28* alleles. Although the protein does not encode a phosphatase, circumstantial evidence suggests that it may affect phosphatase activity in the cell, an activity that could account for its phenotypic effects) (Sutton et al. 1991). Besides these three genes, two other genes encode type 2A homologs, *PPH3* and *PPG1*. Deletion of *PPH3* imparts no phenotype to the cell on its own but renders *pph21 pph22* strains inviable (Ronne et al. 1991). Thus, it partially complements loss of the major 2A species in the cell. *PPG1* is not essential, but deletion of the gene results in diminished accumulation of glycogen (Posas et al. 1993).

We examined the basal and ceramide activated 2A phosphatase activity in strains deleted for each of the above phosphatase genes. The results of this analysis, shown in Figure 5, indicate that Sit4p is a subunit of CAPP. Deletion of *PPH21*, *PPH22*, or both substantially reduces the basal level of unstimulated protein phosphatase 2A activity in the cell, as reported previously. However, substantial ceramide-activated phosphatase activity is still present. In contrast, deletion of *SIT4* does not significantly diminish basal phosphatase activity but almost completely eliminates ceramide stimulated phosphatase activity. Deletion of *PPH3* or *PPG1* has little effect on either the basal or ceramide-stimulated activity (data not shown). Thus, we conclude that Sit4p is the primary catalytic subunit of CAPP. Consistent with this conclusion, we found that Sit4p cofractionated with yeast CAPP over several chromatographic steps, as shown above (Fig. 3). This conclusion is also consistent

with our previous results showing that *sit4* and *tpd3* are synthetically lethal, suggesting that Tpd3p and Sit4p might be involved in a common cellular function (van Zyl et al. 1992). Finally, the conclusion is confirmed by the biological responses of *sit4* strains to ceramide as described below.

Tpd3p, Cdc55p, and Sit4p mediate ceramide-induced growth arrest in yeast

As noted above, Fishbein et al. (1993) demonstrated that ceramide at micromolar levels inhibited proliferation of *Saccharomyces* and that this effect was quite specific for ceramide and its derivatives. We have extended this observation to show that ceramide arrests yeast specifically at the G₁ stage of the cell cycle and that this effect is mediated by Tpd3p, Cdc55p, and Sit4p. These observations, in conjunction with our biochemical studies described above, suggest that a CAPP composed of Tpd3p, Cdc55p, and Sit4p can modulate the G₁ to S transition in yeast.

In Figure 6, we show the effects of ceramide on growth of various yeast strains. In these experiments, cultures were inoculated at 5×10^3 cells/ml in the presence of the indicated concentration of C₂-ceramide and incubated for ~18 hr at 25°C. The concentration of viable cells in the culture at the end of 24 hr is plotted as a function of ceramide concentration. Consistent with the results reported by Fishbein et al. (1993), we find that wild-type strains of yeast are sensitive to ceramide, showing complete inhibition of growth at concentrations of ceramide between 10 and 15 μ M (YCp50-*TPD3* in Fig. 6A, YCp50-*CDC55* in B, and *PPH21 PPH22* in C). In contrast, strains carrying a deletion of *TPD3* (*tpd3::LEU2*, Fig. 6A) or *CDC55* (*cdc55::LEU2*, B) are completely resistant to growth inhibition by C₂-ceramide, whereas isogenic *TPD3* or *CDC55* strains are sensitive. Thus, we find a strict correlation between the absence of CAPP activity (see above) and resistance to ceramide-induced growth inhibition. Fortifying this correlation is the observation that the degree of ceramide-induced inhibition of strains carrying point mutations in *TPD3* or *CDC55* coincides with the level of residual CAPP activity in these strains (cf. *tpd3-1* in Fig. 6A with line 12 in Fig. 5 and *cdc55-1* in Fig. 6B with line 9 in Fig. 5). Thus, we conclude that the growth inhibition induced by ceramide is mediated by the CAPP encoded by *TPD3* and *CDC55*.

We have used ceramide-induced growth inhibition as a biological assay to confirm our assignment of the catalytic subunit of CAPP. As evident in Figure 6C, strains lacking either *PPH21* or *PPH22* or both are as sensitive to ceramide as the isogenic parent strain. Thus, neither of these genes probably encodes the catalytic subunit of CAPP. We also tested the ceramide sensitivity of various *sit4* strains, all of which were viable because they were also *SSD1*. As shown in Figure 6D, a *sit4* deletion strain (*sit4::HIS3*) is completely resistant to ceramide-induced growth inhibition, whereas strains carrying either the conditional *sit4-102* allele or wild-type *SIT4* [YCp50-

Figure 6. Effect of C_2 -ceramide on the growth of various Yeast PP2A wild-type and mutant strains. The indicated yeast strains were inoculated into appropriately supplemented synthetic media at a concentration of 5×10^3 cells/ml and incubated at 25°C in the absence and presence of the indicated concentrations of C_2 -ceramide. For each strain, cultures were grown until the density of the culture lacking ceramide reached $\sim 1 \times 10^7$ cells/ml (~ 18 hr), at which point the density of the ceramide-containing cultures of that strain was determined both by cell counting and plating for viable cells. Both methods gave essentially identical results. All cultures contained an equal concentration of ethanol (0.1% final). For strains in which growth to saturation required substantially >18 hr (*tpd3::LEU2*, *pph21 pph22*, and *sit4::HIS3*), C_2 -ceramide was re-added to the same indicated concentration at 12 hr to compensate for metabolic depletion of the exogenous ceramide (Fishbein et al. 1993). Values represent the data of one of five independent experiments, all of which gave essentially identical results.

SIT4) are sensitive when grown at the permissive temperature. Thus, *SIT4* is required for ceramide-induced inhibition of growth. This confirms our biochemical studies demonstrating that Sit4p is the catalytic subunit of CAPP.

Ceramide induces G_1 arrest of yeast

To evaluate the nature of ceramide-induced growth inhibition of yeast, we examined the morphology of cells following treatment with ceramide. As noted in Figure 7, addition of ceramide to exponentially growing cells results in a dose-dependent accumulation of unbudded cells. Thus, ceramide appears to inhibit growth by inducing cell cycle arrest at the beginning of the cell cycle. Within 4 hr of ceramide addition, $>80\%$ of the cells are unbudded, indicating that arrest occurs within one to two generations. With extended incubation, cells treated with lower concentrations of ceramide resume budding, whereas those treated with higher concentrations remain unbudded. This suggests that cell cycle arrest following treatment with ceramide is completely reversible and that cells are capable either of metabolizing C_2 -ceramide to a noninhibitory species or of adapting to the ceramide-induced signal. Consistent with the fact that *tpd3*, *cdc55*, and *sit4* strains are resistant to growth inhibition by ceramide, these strains do not exhibit an increase in unbudded cells when treated with ceramide, whereas isogenic wild-type strains do (Table 1). Thus,

Tpd3p, Cdc55p, and Sit4p mediate the G_1 arrest induced by ceramide.

The morphology of the arrested cells provides some indication of the target of the ceramide-activated signal.

Figure 7. C_2 -ceramide induces arrest at the beginning of the cell cycle. Strain YCM35 was pregrown in synthetic complete media to 10^7 cells/ml and diluted to 5×10^3 cells/ml in the same media plus the indicated concentration of C_2 -ceramide. Samples were examined microscopically at the indicated time to determine the percentage of budding cells. More than 250 cells were examined for each time point. (●) No added C_2 -ceramide; (○) $5 \mu\text{M}$ C_2 -ceramide; (▲) $10 \mu\text{M}$ C_2 -ceramide; (△) $15 \mu\text{M}$ C_2 -ceramide.

Table 1. Ceramide-induced G_1 arrest requires CAPP

Strain	Percent unbudded cells	
	– C_2 -ceramide	+ C_2 -ceramide
<i>TPD3 CDC55 SIT4</i>	14	82
<i>tpd3Δ</i>	35	42
<i>tpd3Δ</i> [YCp50– <i>TPD3</i>]	10	89
<i>cdc55Δ</i>	12	19
<i>cdc55Δ</i> [YCp50– <i>CDC55</i>]	9	80
<i>sit4Δ</i>	26	33
<i>sit4Δ</i> [YCp50– <i>SIT4</i>]	12	84

Strains were grown at 23°C to 5×10^6 cells/ml in synthetic complete media or synthetic complete lacking uracil for strains bearing YCp plasmids. Cultures were diluted to 5×10^3 in the same media with and without 10 μ M C_2 -ceramide. The percent unbudded cells in each culture was determined after 16 hr incubation at 23°C by microscopic examination.

Cells treated with ceramide-arrested with no bud and a single nucleus, indicating arrest in G_1 prior to bud emergence and initiation of DNA synthesis. This was confirmed by FACS analysis of ceramide-treated cells, demonstrating accumulation of cells with 1N DNA content (Fig. 8). Cells arrested at G_1 with ceramide did not increase in size during the period of arrest, as determined by microscopic observation and by light scattering in FACS analysis (data not shown), nor did they exhibit anisotropic morphological changes associated with the treatment of cells with mating pheromone. This indicates that cells treated with ceramide are blocked at the stage designated as Start II, which is the point of arrest of nutrient-depleted cells or cells deficient in the RAS signal transduction pathway.

The similarity of the phenotypes of ceramide-treated cells and of mutants defective in Ras function prompted us to examine potential interaction between the RAS and ceramide pathways in yeast. In yeast the RAS pathway functions predominantly if not exclusively to activate adenylyl cyclase and, consequently, cAMP-dependent protein kinase (A kinase) (Broach and Deschenes 1990; cf. Fig. 9). Because activation of the ceramide phosphatase yields the same phenotype as loss of activity of the yeast A kinase, we speculated that the role of CAPP might be to antagonize the A kinase. Accordingly, we asked whether elimination of CAPP would exacerbate the phenotype of mutants constitutively activated for the Ras pathway. As shown in Table 2, this is the case. Strains carrying mutations in either the A or B subunit of CAPP could not support propagation of a plasmid carrying an activated allele of *RAS2*, although the same strains would support propagation of a plasmid carrying the wild-type allele of *RAS2*. Thus, activation of the RAS pathway is synthetically lethal with reduced CAPP function, demonstrating a genetic interaction between the two pathways. Similar results were obtained by tetrad analysis of appropriate crosses (data not shown). These results suggest that the RAS and ceramide pathways might affect a common biological process in yeast but with opposite consequences.

Discussion

CAPP and ceramide signaling in yeast

In mammalian cells, ceramide serves as a second messenger in a signal transduction system that induces an antiproliferative effect in response to treatment with several growth modulators. Because okadaic acid blocks the biological responses initiated by these growth modulators, a protein phosphatase 2A species probably plays a critical role in the process. This has been taken as evidence that mammalian CAPP transmits the ceramide-mediated signal. Our results provide a more definitive demonstration that CAPP serves as a critical intermediary in a ceramide signaling pathway. We have shown that ceramide induces cell cycle arrest in yeast and that mutation specifically of the CAPP alleviates ceramide inhibition. Thus, CAPP specifically, and not some other PP2A species, is an intermediary in ceramide signaling. This result in yeast lends credence to the hypothesis that CAPP plays a similar role in signaling in mammalian cells.

Although CAPP is clearly required for eliciting the biological effects of ceramide, it may not be the only critical component of the pathway. We have found that in addition to the CAPP, *Saccharomyces* contains a CAPK (J.T. Nickels and J.R. Broach, unpubl.). Like the mammalian CAPK, the yeast CAPK is membrane associated and proline directed. Accordingly, we cannot conclude that CAPP is the sole mediator of the ceramide response, in either yeast or mammalian cells. For instance, CAPP and CAPK may both be required to manifest the panoply of responses to ceramide activation. In this case, loss of either activity would attenuate the biological response. Alternatively, CAPK may be responsible for a subset of responses that are independent of CAPP but not fully appreciated as downstream effects of ceramide. Molecular cloning of CAPK and characterization of CAPK mutants will be required for a clearer definition of the ceramide pathway.

We have been able to define specifically the compo-

Figure 8. FACS analysis of C_2 -ceramide-treated YCM35 cells. Cells were grown in synthetic media and diluted to 5×10^3 cells/ml. C_2 ceramide (10 μ M) was added, and samples were removed at the time of addition (A) or 2 hr following addition (B). Samples were prepared and analyzed by fluorescence activated cell sorting as described in Materials and methods.

Figure 9. The ceramide signal transduction pathway in yeast. Ceramide produced by an as yet unknown mechanism yields activation of a plasma membrane (PM)-bound kinase (CAPK) and CAPP encoded by *CDC55*, *TPD3*, and *SIT4*. This results in arrest of cells in the G_1 phase of the cell cycle. Although the role of CAPK in the antiproliferative response is unresolved, CAPP probably functions at least in part to dephosphorylate many of the proteins critical for proliferation that are phosphorylated by the cAMP activated kinase (TPK).

nents of the yeast CAPP and show that it is a distinct species from the major 2A phosphatase in the cell. *TPD3* and *CDC55* encode the A and B regulatory subunits, respectively, of CAPP: Anti-Tpd3p antibodies precipitate almost all of CAPP and leave most of the basal 2A activity in the supernatant. In addition, mutational inactivation of either *TPD3* or *CDC55* completely eliminates CAPP activity. Our conclusion that *SIT4* encodes the catalytic subunit is based on two observations. First, *sit4* mutants lack most CAPP activity, whereas *pph21 pph22* mutants retain a significant amount of CAPP activity. Second, *sit4* mutants are completely resistant to ceramide inhibition, whereas *pph21 pph22* strains are as sensitive as wild type. Thus, two distinct lines of evidence suggest that Sit4p is the major catalytic subunit for CAPP. These results are consistent with those of Dobrowsky et al. (1993), who found that, at least in cer-

tain cell types, mammalian CAPP is distinct from the major PP2A species in the cell.

Another interpretation regarding the composition of CAPP is that Tpd3p and Cdc55p are the primary determinants of CAPP activity and that in the presence of ceramide these subunits can draft a variety of different catalytic subunits, including Pph21p, Pph22p, and Sit4p, to constitute ceramide-specific phosphatase activity. This interpretation would account for the fact that we see some residual CAPP activity in *sit4* strains and some diminution of CAPP activity in *pph21 pph22* strains, indicating that Tpd3p and Cdc55p may associate with Pph21p and/or Pph22p to comprise some Sit4p-independent CAPP in yeast. This would also be consistent with the fact that *sit4* and *tpd3* mutations exhibit synthetic lethality (van Zyl et al. 1992): In the absence of Sit4p, Tpd3p could recruit Pph21 or Pph22 for activity, and in the absence of Tpd3p, Sit4p might provide some CAPP activity through interaction with Cdc55p. Thus, both proteins would have to be eliminated to reveal the essential nature of the CAPP. Finally, this interpretation is consistent with prior reports that Tpd3p interacts with Pph21p/Pph22p in the cell. Thus, the subunit composition of the various PP2A species in the cell may be either heterogeneous or dynamic, perhaps changing in response to internal and external cues.

Mechanism of CAPP activation

The effect of eliminating Cdc55p or Tpd3p on CAPP activity described in this paper suggests that rather than functioning as negative regulators of the catalytic subunit, these proteins function as coactivators with ceramide of the catalytic activity of the protein. That is, in

Table 2. Genetic interaction of the RAS and ceramide pathways in yeast.

Strain ^b	Transformation efficiency ^a	
	pRAS2	pRAS2 ^{val19}
<i>TPD3 CDC55</i>	800	2000
<i>cdc55-1</i>	500	<1
<i>tpd3-1</i>	1200	<1

^aThe indicated strains ($\sim 10^7$ competent cells) were transformed to Ura⁺ with 1.0 μ g of DNA of plasmid pRAS2 or pRAS2^{val19}, isogenic *CEN4-URA-3*-based described in Materials and methods. Values are the calculated number of Ura⁺ colonies/ μ g of DNA obtained after 4 days incubation at 23°C following transformation.

^bStrains are listed in Table 3.

the context of existing data on PP2A structure and function [Mumby and Walter 1993], we might have anticipated that Cdc55p and/or Tpd3p would act to inhibit the catalytic activity of the cognate C subunit and that ceramide would activate CAPP by alleviating that inhibition. However, if this were the case, then loss of either Cdc55p or Tpd3p would yield phosphatase levels equivalent to that obtained in the presence of ceramide. Instead, we find that the level of phosphatase activity in the absence of Cdc55p or Tpd3p is equivalent to that obtained in the absence of ceramide. Therefore, we conclude that Cdc55p and/or Tpd3p functions in the presence of ceramide to activate the catalytic subunit. That is, one or both of these subunits are coactivators with ceramide of CAPP.

Previous work by Dobrowsky et al. [1993] suggested that the effect of ceramide was mediated by the B subunit of a phosphatase 2A. This conclusion was based on the observation that PP2A activity fractionating as an ABC heterotrimer was ceramide responsive but PP2A activity fractionating as an AC heterodimer was not ceramide responsive. In contrast, as noted above, our results suggest that both the A and B subunits are required for ceramide activation of the yeast CAPP. We can reconcile these observations by suggesting that Cdc55p, the B subunit cognate in yeast, actually mediates ceramide activation but that the A subunit, Tpd3p, is required for association of the B regulatory subunit with the C catalytic subunit. Thus, the absence of observable CAPP activity in a *tpd3* strain is attributable to the fact that, in the absence of Tpd3p, Cdc55p cannot associate with the catalytic subunit to stimulate its activity. Consistent with this interpretation, fractionation studies of mammalian PP2A have demonstrated that the protein can exist as a stable heterotrimer comprised of the A, B, and C subunits or as a heterodimer comprised of the A and C subunits but that stable BC heterodimers are never found [Cohen 1989]. Thus, B subunits of the PP2A family appear to require an A subunit to associate with a catalytic subunit, and we would suggest that the same is true for yeast CAPP.

Our results cannot be readily reconciled with a recent report suggesting that the catalytic subunit of CAPP could respond directly to ceramide [Law and Rossie 1995]. We find that strains containing the catalytic subunit of CAPP but lacking one or the other regulatory subunit fail to exhibit ceramide-induced phosphatase activity. Thus, the yeast CAPP catalytic subunit is not capable of direct activation by ceramide.

Potential upstream signals and Ddownstream targets of CAPP in yeast

The presence of both a CAPP and a CAPK and the ability of ceramide to induce a pronounced biological effect argue for the existence of a signal transduction system in yeast mediated by ceramide and CAPP. As yet lacking from this pathway, though, are the identity of the initiating signal and the nature of the downstream targets.

How might ceramide signaling be initiated in yeast? In

mammalian cells, ceramide as second messenger is derived from sphingomyelin by a ligand-inducible sphingomyelinase. In a similar vein, ceramide signaling might be initiated in yeast by activation of sphingolipid hydrolysis in response to internal or external stimuli. The genetic interaction between *cdc55* and *elm1*, a mutation promoting the pseudohyphal morphology elicited by nitrogen starvation of certain strains, might suggest a role for nitrogen depletion as a stimulus for ceramide signaling [Blacketer et al. 1993]. However, insufficient information is currently available to assess whether nitrogen availability or any other external stimuli can affect ceramide levels to initiate ceramide signaling in yeast. An intriguing alternative possibility is that ceramide signaling responds to internal cues, such as free ceramide generated during biosynthesis of sphingolipids in yeast. Ceramide is synthesized by condensation of sphinganine with a fatty acid and is then converted to sphingolipid by condensation with inositol-phosphate. We might speculate that the ceramide-activated pathway exists in yeast to monitor free ceramide in this biosynthetic process as a means of coupling the continuous process of lipid production to the discontinuous process of cell cycle progression.

The relevant downstream targets of CAPP in cell cycle regulation are not known, although genetic analyses of mutants defective in components of yeast CAPP and the terminal phenotype of ceramide-treated cells provide material for speculation. In particular, we find that the phenotype of cells treated with ceramide is identical to that obtained with cells with reduced *RAS* activity. In addition, we find that strains lacking CAPP activity cannot support activation of the *RAS* pathway. Sutton et al. [1991] reported a similar genetic interaction in noting that *sit4* dramatically exacerbated the activated phenotypes of a *bcy1* strain. Accordingly, we propose that some of the targets of CAPP may be those proteins involved in growth control that are phosphorylated and activated by the A kinase. Thus, activation of the *RAS* pathway in strains lacking CAPP would drive these target proteins into a hyperphosphorylated state, which would be lethal. This is consistent with the fact that hyperactivation of the *RAS* pathway in yeast results in cell inviability [Fedor-Chaiken et al. 1990]. Although these critical target proteins in growth regulation have not been identified, the relationship between ceramide signaling and the *RAS* pathway should facilitate their identification.

Although the connection between ceramide signaling and *RAS* is compelling, antagonism of the A kinase may not be the only role of ceramide signaling in growth regulation. Studies conducted by Arndt and his colleagues have shown that inactivation of *SIT4* results in a block in cell cycle progression at the G_1/S boundary [Sutton et al. 1991] as a result of a requirement for *SIT4* for normal expression of the G_1 cyclin genes *CLN1* and *CLN2* [Fernandez-Sarabia et al. 1992]. Thus, it is possible that the effects of activated CAPP on cell cycle progression may also involve modulation of *CLN* expression or activity. We note, though, that the effects Sutton et al.

(1991) were examining resulted from reduced Sit4p activity, whereas those associated with ceramide treatment would result from enhanced Sit4p activity. Accordingly, we have no reason to suppose that these two conditions would affect the same downstream targets. Nonetheless, the effect of ceramide on expression of the *CLN* genes is an obvious avenue for further investigation.

Homology between yeast and mammalian ceramide signaling

The yeast ceramide pathway is remarkably similar to that of mammalian cells. In mammalian cells, the biological responses elicited by the ceramide-mediated pathway are antiproliferative: terminal differentiation of myeloid cells and induction of G_1 arrest and apoptosis of fibrosarcoma and leukemia cells, for example. In yeast, ceramide also elicits an antiproliferative response: arrest at the G_1 stage of the cell cycle. In both yeast and mammalian cells, this antiproliferative effect requires the activity of a CAPP, which in both cases is a member of the PP2A family. Finally, in both cases, cells contain a membrane-bound proline-directed CAPK, whose role in the signaling pathway remains elusive.

Whereas studies of the ceramide pathway in mammalian cells will probably continue apace, studies of this pathway in yeast offer a novel and complementary approach to dissecting this signal transduction pathway. One obvious direction is the use of yeast mutants as a means of recovering the mammalian genes encoding specific components of the pathway. That is, complementation of mutants in the yeast CAPP or CAPK with mammalian cDNA clones may prove to be the most effective means of recovering and characterizing these genes, much in the way Martegani et al. (1992) were able to isolate a mammalian guanine nucleotide exchange fac-

tor gene by complementing a yeast *cdc25* mutant. At the very least, though, dissection of the pathway should provide valuable insight into the mechanism by which mammalian cells limit their proliferation, the lessons from which should have profound implications in understanding cell growth and its control.

Materials and methods

Strains

All strains used in this study are listed in Table 3, along with their sources. Strains Y2010, Y2011, and Y2014 were obtained by transforming strains Y1370, AHY86, and CY279 to uracil prototrophy with plasmids YCp50-*TPD3*, YCp50-*CDC55*, and YCp50-*SIT4*, respectively. Plasmids pRAS and pRAS^{val19} are isogenic *CEN4-URA3*-based plasmids constructed by cloning the *HindIII-EcoRI* fragments spanning *RAS2* and *RAS2*^{val19} from plasmids pRJ4-27 and pMF100 (Fedor-Chaiken et al. 1990), respectively, into vector YCp50.

Strains were grown as indicated either in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic complete media (Sherman et al. 1982) containing 0.67% yeast nitrogen base, supplemented appropriately with amino acids, adenine, and uracil. For studies of the effects of ceramide on cell growth, single colonies were inoculated into the appropriate media and grown overnight to a cell number of 0.5×10^7 to cells/ml. Aliquots of each culture were then diluted into fresh media at a cell number of 5×10^3 cells/ml and grown in the absence and presence of the indicated concentrations of C_2 -ceramide (obtained from Matreya Corp., Pleasant Gap, PA). Growth studies requiring time periods longer than 12 hr were supplemented with additional C_2 -ceramide to compensate for any metabolism. C_2 -ceramide was dissolved in ethanol, and the final concentration of ethanol in each culture was 0.1%. Cell growth was determined by manual cell counting using a hemocytometer. Cell viability was determined by plate assay and scored after 3 days. Even at the highest ceramide concentration, cell number and colony forming units were essentially identical.

Table 3. Strains used in this study

Strain	Genotype	Source
YCM35	α <i>pep4::LEU2 ura3-52 leu2-3,112</i>	laboratory stock
W303-1A	α <i>leu2-3,112 trp1-1 ura3-1 his3-11,15</i>	laboratory stock
S288C	α <i>CUP 1 suc 2</i>	laboratory stock
Y1367	α <i>his3Δ1 lys2-1 leu2-3,112</i>	laboratory stock
Y1459	α <i>hml::SUP4 hmr::SUC2 sir4-1 leu2-3,112 his3Δ1 ade2-1 ura3-52 tpd3-1</i>	laboratory stock
Y1370	α <i>his3Δ1 lys2-1 leu2-3,112 ura3-52 tpd3Δ::LEU2</i>	laboratory stock
Y2010	α <i>his3Δ1 lys2-1 leu2-3,112 ura3-52 tpd3Δ::LEU2</i> [YCp50- <i>TPD3</i>]	this study
AHY38	α <i>ura3-52 leu2-3,112 cdc55-1</i>	Healy et al. (1991)
AHY86	α <i>ura3-52 leu2-3,112 cdc55Δ::LEU2</i>	Healy et al. (1991)
Y2011	α <i>ura3-52 leu2-3,112 cdc55Δ::LEU2</i> [YCp50- <i>CDC55</i>]	this study
CY279	α <i>ura3-52 leu2-3,112 trp1-1 his3Δ1 sit4::HIS3 ssd1-dl</i>	Sutton et al. (1991)
Y2014	α <i>ura3-52 leu2-3,112 trp1-1 his3Δ1 sit4::HIS3 ssd1-dl</i> [YCp50- <i>SIT4</i>]	this study
AS3/6-29	α <i>leu2-3,112 ura3-52 his3Δ1 PPH21 PPH22</i>	Sneddon et al. (1990)
AS3/6-30	α <i>leu2-3,112 ura3-52 PPH21 pph22::URA3</i>	Sneddon et al. (1990)
AS3/6-31	α <i>leu2-3,112 ura3-52 his3Δ1 trp1-1 PPH22 pph21::LEU2</i>	Sneddon et al. (1990)
X24-16B	α <i>leu2-3,112 his3Δ1 trp1-1 ade2-1 pph21::LEU2 pph22::HIS3</i>	this study
Y1985	α <i>leu2-3,112 trp1-1 ura3-1 his3-11,15 ppg::TRP1</i>	Posas et al. (1993)
H283	α <i>leu2-3,112 trp1-1 ura3-1 his3-11,15 pph3::URA3</i>	Ronne et al. (1991)

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Preparation of [³²P]phosphohistone

[³²P]Phosphohistone was prepared using cAMP-dependent protein kinase as described previously (Meisler and Langan 1969) with some modifications. Briefly, the reaction was terminated by applying the sample to two successive Bio-Gel P-6 spin chromatography columns that had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The columns were centrifuged at 1000g for 5 min at room temperature. An aliquot of the final column effluent was subjected to analysis by SDS-polyacrylamide gel electrophoresis using 10% slab gels followed by autoradiography. Radiolabeled histone was aliquoted and stored at -80°C and used within 3 days.

Protein phosphatase assay

Phosphatase activity was measured for 15 min at 30°C by following the dephosphorylation of [³²P]phosphohistone (55,000 cpm/nmole) in the absence and presence of C₂-ceramide. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 2.0 mM histone, 5 ng of cAMP-dependent protein kinase inhibitor, and 100–250 µg of protein in a total volume of 0.1 ml. The reaction was terminated and phosphatase activity determined as described previously [Dobrowsky and Hannun 1992]. All assays were performed in triplicate with an average standard deviation of ±5%. All assays were linear with time and protein concentration. [³²P]phosphohistone hydrolysis did not exceed 5 to 10% of the total radiolabel added. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 nmole Pi /min. Specific activity was defined in units per milligram of protein. CAPP activity was calculated by subtracting basal PP2A activity from ceramide-stimulated CAPP activity. Okadaic acid was dissolved in dimethylsulfoxide [DMSO].

Preparation of cytosolic extract

Cytosolic extracts for CAPP purification were prepared from 50 grams [wet weight] of cells by disruption at 4°C with glass beads in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.3 M sucrose, 1 mM PMSF, 1 mM benzamidine, and CLAP protease cocktail (2.5 µg/ml of chymostatin, 2.5 µg/ml of leupeptin, 2.5 µg/ml of antipain, 2.5 µg/ml of pepstatin) using a Biospec Bead Beater (Bartlesville, OK). Cell debris was removed by low-speed centrifugation (3000g), and the crude cellular extract was subjected to differential centrifugation at 100,000g to obtain the cytosolic fraction. The cytosol fraction was brought to 15% glycerol and was used immediately owing to the lability of CAPP activity. Small-scale cytosolic extract preparation (500-ml cultures) was performed under similar conditions using a Biospec mini-Bead Beater.

Partial purification of CAPP

All steps were performed at 4°C. A DE52 column (2.5 x 8 cm) was equilibrated with buffer A (50 mM Tris-HCl at pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol, 15% glycerol, 1 mM PMSF, 1 mM benzamidine, CLAP protease cocktail). The cytosolic extract was applied to the column followed by washing the column with 5 column volumes of buffer A. CAPP activity was eluted from the column with 10 column volumes of a linear NaCl gradient (0–0.5 M) in buffer A at a flow rate of 20 ml/hr. Fractions containing CAPP activity were pooled, diluted threefold with buffer A, and immediately applied to a Mono-Q column (0.5x5 cm) that had been equilibrated with buffer A. The column was washed with 10 column volumes of buffer A fol-

lowed by elution of CAPP activity with 20 column volumes of a linear NaCl gradient (0.1–0.4 M) in buffer A at a flow rate of 30 ml/hr. Fractions containing CAPP activity were pooled, diluted as above, and immediately applied to a polylysine-agarose column (2.5x10 cm) previously equilibrated with buffer A. The column was washed with 5 column volumes of buffer A followed by elution of CAPP activity using 15 column volumes of a linear NaCl gradient (0–0.4 M) in buffer A at a flow rate of 15 ml/hr. Owing to the lability of CAPP activity, no further purification was attempted. All fractions were brought to 20% glycerol and stored at -80°C.

Western analysis and immunoprecipitation of CAPP activity

Tpd3p antibodies were raised against a *trpE-TPD3* fusion protein as described previously [van Zyl et al. 1992]. The IgG fraction was isolated from antisera by DEAE-Affi-Gel Blue chromatography followed by ammonium sulfate precipitation (50% saturation) and dialysis against 50 mM Tris-HCl Buffer (pH 7.4) containing 0.05% NaN₃. Sit4p antisera was a kind gift from Dr. Ann Sutton and was prepared as described previously [Sutton et al. 1991]. For Western analysis of various CAPP chromatography fractions, proteins were resolved by SDS PAGE under denaturing conditions using 10% slab gels, transferred to PVDF membranes, and probed with either anti-Tpd3p IgG antibodies (1:2500 dilution) or Sit4p antisera (1:500 dilution). Blots were then incubated with biotinylated goat anti-rabbit IgG (1:3000 dilution). Tpd3p and Sit4p antibody binding was detected with streptavidin/alkaline phosphatase (1:2000 dilution). Alkaline phosphatase activity was detected using the *p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate chromogenic substrate.

All immunoprecipitations were performed using small-scale cytosolic preparations. Cytosolic extracts (80–375 µg) were incubated with the indicated concentrations of Tpd3p IgG antibodies in buffer B (50 mM Tris-HCl at pH 7.4, 1 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine, 2xCLAP protease cocktail) for 2 hr at 4°C. Twenty microliters of 50% protein A-Sepharose was then added, and the extracts were further incubated for an additional 2 hr at 4°C. Immune complexes were recovered by microcentrifugation, and the pellets were washed twice with high salt buffer (25 mM Tris-HCl at pH 7.4, 250 mM NaCl, 1 mg/ml of BSA) and once with buffer B without protease inhibitors. PP2A and CAPP activities were assayed as indicated in the figure legends.

Microscopy procedures

Cultures were harvested by centrifugation and washed twice in buffer C (50 mM KPO₄ at pH 7.0, 250 mM NaCl). Cells were then fixed in methanol, acetic acid (75:25), for 40 min at 0°C. Cells were washed as above and resuspended in buffer C containing 1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) and incubated for 30 min at 0°C. Stained cells were washed as above and mounted onto microscopic slides, and photomicrographs were taken using a Zeiss Axiomat microscope with a Pan-Neofluar 100x/1.3 objective.

FACS analysis

Yeast cells were prepared for flow cytometry using a method modified from Hutter and Eipel [1978]. Cultures were grown in YPD to 1x10⁷ cells/ml, and a sample (1 ml) was harvested by centrifugation, washed with 0.2 M Tris-HCl, (pH 7.5), and fixed in 70% (vol/vol) ethanol for 1 hr at room temperature. Fixed cells were harvested by centrifugation, washed with 0.2 M Tris-

HCl, (pH 7.5), and resuspended in Tris buffer containing 1 mg/ml of RNase A. After 1 hr. at 37°C, cells were harvested, washed with Tris buffer, and resuspended in a fresh solution of 0.5% pepsin in 0.55 N HCl. After 5 min at room temperature, cells were harvested, washed in Tris buffer, and resuspended in 0.18 M Tris-HCl (pH 7.5), 0.19 M NaCl, 70 mM MgCl₂, 50 µg/ml propidium iodide. FACS analysis was performed on a Coulter Electronic Epics 753 cell sorter equipped with narrow beam optics for light scattering and a 570-nm-long pass filter for propidium iodide detection.

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References

- Arndt, K.T., C.A. Styles, and G.R. Fink. 1989. A suppressor of a HIS4 transcription defect encodes a protein with homology to the catalytic subunit of protein phosphatases. *Cell* **56**: 527–537.
- Bielawska, A., C.M. Linardic, and Y.A. Hannun. 1992a. Ceramide-mediated biology. Determination of structural and stereospecific requirements through the use of N-acyl-phenylaminoalcohol analogs. *J. Biol. Chem.* **267**: 18493–18497.
- . 1992b. Modulation of cell growth and differentiation by ceramide. *FEBS Lett.* **307**: 211–214.
- Bielawska, A., H.M. Crane, D. Liotta, L.M. Obeid, and Y.A. Hannun. 1993. Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. *J. Biol. Chem.* **268**: 26226–26232.
- Blacketer, M.J., C.M. Koehler, S.G. Coats, A.M. Myers, and P. Madaule. 1993. Regulation of dimorphism in *Saccharomyces cerevisiae*: Involvement of the novel protein kinase Elm1p and protein phosphatase 2A. *Mol. Cell. Biol.* **13**: 5567–5581.
- Broach, J.R. and R.J. Deschenes. 1990. The function of RAS genes in *Saccharomyces cerevisiae*. *Recent Adv. Cancer Res.* **54**: 79–139.
- Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**: 453–508.
- Dbaiho, G.S., M.Y. Pushkareva, S. Jayadev, J.K. Schwarz, J.M. Horowitz, L.M. Obeid, and Y.A. Hannun. 1995. Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest. *Proc. Natl. Acad. Sci.* **92**: 1347–1351.
- Dobrowsky, R.T. and Y.A. Hannun. 1992. Ceramide stimulates a cytosolic protein phosphatase. *J. Biol. Chem.* **267**: 5048–5051.
- Dobrowsky, R.T., C. Kamibayashi, M.C. Mumby, and Y.A. Hannun. 1993. Ceramide activates heterotrimeric protein phosphatase 2A. *J. Biol. Chem.* **268**: 15523–15530.
- Dressler, K.A., S. Mathias, and R.N. Kolesnick. 1992. Tumor necrosis factor- α activates the sphingomyelin signal transduction pathway in a cell-free system. *Science* **255**: 1715–1718.
- Fedor-Chaikin, M., R.J. Deschenes, and J.R. Broach. 1990. SRV2, a gene required for RAS activation of adenylate cyclase in yeast. *Cell* **61**: 329–340.
- Fernandez-Sarabia, M.J., A. Sutton, T. Zhong, and K.T. Arnot. 1992. SIT4 protein phosphatase is required for the normal accumulation of SWI4, CLN1, CLN2, and HCS26 RNAs during late G₁. *Genes & Dev.* **6**: 2417–2428.
- Fishbein, J.D., R.T. Dobrowsky, A. Bielawska, S. Garrett, Y.A. Hannun. 1993. Ceramide-mediated growth inhibition and CAPP are conserved in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**: 9255–9261.
- Hannun, Y.A. 1994. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* **269**: 3125–3128.
- Hannun, Y.A. and R.M. Bell. 1993. The sphingomyelin cycle: A prototypic sphingolipid signaling pathway. *Adv. Lipid Res.* **25**: 27–41.
- Hannun, Y.A., L.M. Obeid, and R.A. Wolff. 1993. The novel second messenger ceramide: Identification, mechanism of action, and cellular activity. *Adv. Lipid Res.* **25**: 43–64.
- Healy, A.M., S. Zolnierowicz, A.E. Stapleton, M. Goebel, A. DePaoli-Roach, and J.R. Pringle. 1991. CDC55, a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: Identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. *Mol. Cell. Biol.* **11**: 5767–5780.
- Heller, R.A. and M. Kronke. 1994. Tumor necrosis factor receptor-mediated signaling pathways. *J. Cell Biol.* **126**: 5–9.
- Hutter, K.J. and H.E. Eipel. 1978. Flow cytometric determinations of cellular substances in algae, molds and yeast. *Antonie Leeuwenhoek J. Microbiol.* **44**: 269–282.
- Jarvis, W.D., R.N. Kolesnick, R.A. Fornari, R.S. Traylor, D.A. Gewitz, and S. Grant. 1994. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. *Proc. Natl. Acad. Sci.* **91**: 73–77.
- Jayadev, S., B. Liu, A.E. Bielawska, J.Y. Lee, F. Nazaire, M.Y. Pushkareva, L.M. Obeid, and Y.A. Hannun. 1995. Role for ceramide in cell cycle arrest. *J. Biol. Chem.* **270**: 2047–2052.
- Kolesnick, R.N. 1991. Sphingomyelin and derivatives as cellular signals. *Prog. Lipid Res.* **30**: 1–38.
- . 1992. Ceramide: A novel second messenger. *Trends Cell Biol.* **2**: 233–236.
- Kolesnick, R. and D.W. Golde. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77**: 325–328.
- Law, B. and S. Rossie. 1995. The dimeric and catalytic forms of protein phosphatase 2A from rat brain are stimulated by C2-ceramide. *J. Biol. Chem.* **270**: 12808–12813.
- Lester, R.L. and R.C. Dickson. 1993. Sphingolipids with inositolphosphate-containing head groups. *Adv. Lipid Res.* **26**: 253–273.
- Liu, J., S. Mathias, Z. Yang, and R.N. Kolesnick. 1994. Renaturation and tumor necrosis factor- α stimulation of a 97 kDa ceramide activated protein kinase. *J. Biol. Chem.* **269**: 3047–3052.
- Martegani, E., M. Vanoni, R. Zippel, P. Coccetti, R. Brambilla, C. Ferrari, E. Sturani, and L. Alberghina. 1992. Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a *Saccharomyces cerevisiae* RAS activator. *EMBO J.* **11**: 2151–2157.
- Mathias, S., A. Younes, C.-C. Kan, I. Orlov, C. Joseph, and R.N.

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- Kolesnick. 1993. Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 β . *Science* **259**: 519–522.
- Meisler, M.H. and T.A. Langan. 1969. Characterization of a phosphatase specific for phosphorylated histones and protamines. *J. Biol. Chem.* **244**: 4961–4968.
- Mumby, M.C. and G. Walter. 1993. Protein serine/threonine phosphatases: Structure, regulation, and function in cell growth. *Physiol. Rev.* **73**: 673–699.
- Obeid, L.M., C.M. Linardic, L.A. Karolak, and Y.A. Hannun. 1993. Programmed cell death induced by ceramide. *Science* **259**: 1769–1771.
- Okazaki, T., A. Bielawska, R.M. Bell, and Y.A. Hannun. 1990. Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. *J. Biol. Chem.* **265**: 15823–15831.
- Patton, J.L. and R.L. Lester. 1991. The phosphoinositol sphingolipids of *S. cerevisiae* are highly localized in the plasma membrane. *J. Bacteriol.* **173**: 3101–3108.
- Posas, F., J. Clotet, M.T. Muns, J. Corominas, A. Casamayor, and J. Arino. 1993. The gene PPG encodes a novel yeast protein phosphatase involved in glycogen accumulation. *J. Biol. Chem.* **268**: 1349–1354.
- Ronne, H., M. Carlberg, G.Z. Hu, and J.O. Nehlin. 1991. Protein phosphatase 2A in *Saccharomyces cerevisiae*: Effects on cell growth and bud morphogenesis. *Mol. Cell. Biol.* **11**: 4876–4884.
- Sherman, F., G.R. Fink, and J.B. Hicks. 1982. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sneddon, A.A., P.T.W. Cohen, and M.J.R. Stark. 1990. *S. cerevisiae* protein phosphatase 2A performs an essential cellular function and is encoded by two genes. *EMBO J.* **9**: 4339–4346.
- Sutton, A., D. Immanuel, and K.T. Arndt. 1991. The SIT4 protein phosphatase functions in late G1 for progression into S phase. *Mol. Cell. Biol.* **11**: 2133–2148.
- van Zyl, W.H., N. Wills, and J.R. Broach. 1989. A general screen for mutants of *Saccharomyces cerevisiae* deficient in tRNA biosynthesis. *Genetics* **123**: 55–68.
- van Zyl, W., W. Huang, A.A. Sneddon, M. Stark, S. Camier, M. Werner, C. Marck, A. Sentenac, and J.R. Broach. 1992. Inactivation of the protein phosphatase 2A regulatory subunit A results in morphological and transcriptional defects in yeast. *Mol. Cell. Biol.* **12**: 4946–4959.
- Wells, G.B. and R.L. Lester. 1983. The isolation and characterization of a mutant strain of *S. cerevisiae* that requires a long chain base for growth and for synthesis of phosphosphingolipids. *J. Biol. Chem.* **258**: 10200–10203.
- Yanaga, F. and S.P. Watson. 1992. Tumor necrosis factor α stimulates sphingomyelinase through the 55 kDa receptor in HL-60 cells. *FEBS Lett.* **314**: 297–300.

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References

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