Evidence for Antagonistic Regulation of Cell Growth by the Calcineurin and High Osmolarity Glycerol Pathways in *Saccharomyces cerevisiae*  

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Because Ca\(^{2+}\) signaling of budding yeast, through the activation of calcineurin and the Mpk1/Slt2 mitogen-activated protein kinase cascade, performs redundant function(s) in the events essential for growth, the simultaneous deletion of both these pathways (Δcnb1 Δmpk1) leads to lethality. A PTC4 cDNA that encodes a protein phosphatase belonging to the PP2C family was obtained as a high dosage suppressor of the lethality of Δcnb1 Δmpk1 strain. Overexpression of PTC4 led to a decrease in the high osmolarity-induced Hog1 phosphatase, and HOG1 deletion remarkably suppressed the synthetic lethality, indicating an antagonistic role of the high osmolarity glycerol (HOG) pathway and the Ca\(^{2+}\) signaling pathway in growth regulation. The calcineurin-Crz1 pathway was required for the down-regulation of the HOG pathway. Analysis of the time course of actin polarization, bud formation, and the onset of mitosis in synchronous cell cultures demonstrated that calcineurin negatively regulates actin polarization at the bud site, whereas the HOG pathway positively regulates bud formation at a later step after actin has polarized.

Cells of the yeast *Saccharomyces cerevisiae* evaluate and cope with their external environment by using rapidly responding, highly complex signaling pathways. Of particular importance are the signaling pathways known collectively as the mitogen-activated protein kinase (MAPK) cascade, which couples an extracellular input signal to a variety of outputs. The budding yeast possesses five MAPKs acting in five functionally distinct cascades, each mediating physiologically distinct responses (1). Adaptation to high osmolarity is mediated by the HOG (high osmolarity glycerol) pathway, in which the Hog1 MAPK cascade alone does not lead to lethality under normal conditions, simultaneous defects of these pathways are synthetically lethal, indicating that the two pathways perform redundant function(s) in same essential event(s) for cell growth (19, 20). Upon activation by Ca\(^{2+}\) or various stimuli, the Mpk1 MAPK cascade maintains cell wall integrity by controlling the cell wall (1). In addition to these mechanisms, the two signaling pathways coordinately regulate the G1-M transition through the activation of Swe1, a negative regulator of Cdc28/Cib in the G2 phase (21). In this process, calcineurin up-regulates Swe1 function in two ways, one by the activation of the transcription of SWE1 gene and the other by the destabilization of Hsl1 kinase, a negative regulator of Swe1 kinase. High concentration of CaCl\(_2\) in the medium causes the cells to activate these pathways and leads the cells to a G2 cell-cycle delay. It was proposed that the activation of these pathways occurs in response to a membrane stretch stress (21).

In contrast to the HOG pathway, the Pkc1-Mpk1/Slt2 MAPK cascade responds to low osmolarity conditions. An important function of the Pkc1 pathway is to maintain cell wall integrity in response to low osmolarity and high temperature conditions by controlling the assembly of the cell wall (1). Another major mechanism for adaptation to environmental changes in yeast is Ca\(^{2+}\) signaling. Calcineurin, essential only under specific environmental conditions, is an important mediator of the Ca\(^{2+}\) signal. Calcineurin is required for the recovery from the G0 arrest induced by mating pheromones and for homeostasis of ions, including Na\(^+\)/Li\(^+\), Mn\(^{2+}\), and OH\(^-\) (19–21). Calcineurin regulates the expression of ion transporter genes by a mechanism mediated by Crz1, a calcineurin-dependent transcription factor (17, 18). Although the defect of either calcineurin or the Mpk1 MAPK cascade alone does not lead to lethality under normal conditions, simultaneous defects of these pathways are synthetically lethal, indicating that the two pathways perform redundant function(s) in same essential event(s) for cell growth (19, 20). The activation of Swe1, a negative regulator of Clb in the G2-M transition (21). In this process, calcineurin up-regulates Swe1 function in two ways, one by the activation of the transcription of SWE1 gene and the other by the destabilization of Hsl1 kinase, a negative regulator of Swe1 kinase. High concentration of CaCl\(_2\) in the medium causes the cells to activate these pathways and leads the cells to a G2 cell-cycle delay. It was proposed that the activation of these pathways occurs in response to a membrane stretch stress (21).

In this present report, we show that the HOG pathway and the Ca\(^{2+}\) signaling pathways antagonize one another in the regulation of cell growth. We further demonstrate that these pathways are antagonistic in the regulation of bud emergence. In the presence of CaCl\(_2\) in the medium, the activation of
calcineurin negatively regulates actin polarization at the bud site, whereas the HOG pathway promotes bud formation at a step after actin polarization. We also show that the calcineurin-Crz1 pathway is involved in the down-regulation of the HOG pathway activated by Ca\textsuperscript{2+} or hyperosmotic stress.

**MATERIALS AND METHODS**

**Yeast Strains, Media, and Plasmids**—Yeast strains used in this study are listed in Table I. All yeast strains were derivatives of W303-1A. Media used were as described previously (21). PBS2 and SSK1 was deleted with the pbs2::URA3 and ssh1::URA3 fragment from pPB22 and pDDS12 respectively (gift from T. Maeda). STE11 and pDSS12 respectively (gift from T. Maeda). galactose-inducible ste11 was converted to a XhoI site using the linker oligonucleotide YEplac195GAL-PTC1, YEplac112GAL-PTC2, and YEplac112GAL-PTC2, and cloned into NheI/XbaI-digested YEp51 vector.

**Strain Genotype Source**

| Strain | Genotype | Source |
|--------|----------|--------|
| MATa ade2-1 his3-11, 15 leu2-3, 112 trpl-1::ura3-1 can1-100 | From Dr Rothstein | |
Ca$^{2+}$ Signal and HOG Pathway Act Antagonistically

RESULTS

Identification of PTC4 cDNA as a High Dosage Suppressor of the Synthetic Lethality of the Δcnb1 Δmpk1 Double Deletion To clarify the cellular event(s) that are regulated by the Ca$^{2+}$ signaling pathways (calcineurin and Mpk1 mitogen-activated protein kinase cascade), we screened for the complementary DNA (cDNA), overexpression of which could rescue the synthetic lethality of the Δcnb1 Δmpk1 strain. Because the double deletion mutant can grow in the presence of an osmotic selection medium containing 1 M sorbitol at 28 °C and shifted to 30 °C medium without sorbitol. Hog1 protein was immunoprecipitated by anti-HA antibody (HA.11, Sigma) for 90 min at 4 °C. The immunoprecipitates were washed once with buffer A, twice with buffer B (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 5 mM MgCl$_2$, 1 mM DTT, 10 mM sodium vanadate, 10 mM NaF, and 1 mM PMSF), and once with kinase assay buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EGTA, 5 mM MgCl$_2$, 1 mM DTT, 1 mM sodium vanadate). The 25-μM supernatants of immunoprecipitates were used for immunoblotting as a loading control. To assay Hog1 kinase activity, Hog1-HA bound to protein A beads was incubated with γ-[32P]ATP (5 μCi) and 45 μg of myelin basic protein (MBP, Sigma) for 30 min at 30 °C. Incorporation of 32P into MBP was assessed by SDS-PAGE and quantified by BAS-1800 Bioimaging analyzer. In the experiments shown in Fig. 2, Δcnb1 Δmpk1 strain (TNF36) was transformed with vector only, pGAL-PTC4 (pNV7-PTC4), or pGAL-PTP2 (pSSP25, Ref. 3). The cells were grown to 6–8 × 10$^6$ cells/ml in SR minus uracil medium containing 1 M sorbitol at 28 °C and shifted to 30 °C medium without sorbitol. Hog1 protein was immunoprecipitated by anti-Hog1 antibody (y-215, Santa Cruz Biotechnology) with protein A beads and assayed for kinase activity as described above.

Fluorescence-activated Cell Sorting Analysis, Fluorescence Staining, and Microscopy—Approximately 1 × 10$^6$ cells from yeast culture were harvested by centrifugation and resuspended in 300 μl of 0.2 M Tris-HCl (pH 7.5). Cells were fixed by addition of 700 μl of cold ethanol gradually with vortexing, followed by incubation for overnight at −20 °C. The fixed cells were washed with the same buffer containing RNase A (1 mg/ml) for 3 h at 30 °C. The cells were stained with 100 μl of propidium iodide (50 μg/ml) solution in 4 mM sodium citrate, 10 mM NaCl, and 0.1% Nonidet P-40 for 15 min on ice. The cells were analyzed by FACScalibur (Becton Dickinson). F-actin was visualized by staining with rhodamine-conjugated phalloidin, as described (24). Quantification of cumulative percentage of bud formation and nuclear division during cell cycle progression in various strains that were synchronized with α-factor was determined by microscopic observation and propidium iodide staining. At least 300 cells were counted for each time point.

PTC4 overexpression suppresses the lethality of the Δcnb1 Δmpk1 strain by negatively regulating Hog1 MAPK. A, effect of Hog1 deletion (Δhog1) on the growth of Δcnb1 Δmpk1 strain. WT (W303), Hog1 (SOSS), Δcnb1 (DTT14), Δmpk1 (TNF46), Δcnb1 Δmpk1 (TNF36), or Δhog1 Δcnb1 Δmpk1 (SOS48) cells were spotted on agar plates of YPD, or YPD + 1 M sorbitol (+Sor), and cultured for 2 days at the indicated temperature. B, effect of PTC4 overexpression on the high osmolarity-induced Hog1 phosphorylation. WT (W303) cells transformed with YEp51 (vector) or YEp51-PTC4 (pGAL-PTC4) were grown in SR minus leucine (same composition as SD minus leucine, except 2% raffinose was used instead of dextrose) to early log phase and shifted to 30 °C (WT) or to 37 °C (vector). Whole-cell extracts were prepared, and immunoblotting was performed with anti-phospho-p38 or anti-HA as a loading control. C, Hog1 kinase activity was measured using the same cell extracts. Immunoprecipitated Hog1-HA was incubated with MBP and γ-[32P]ATP. The level of Hog1-HA in lysate was assessed by immunoblotting with anti-HA antibody. The radiolabel incorporated into MBP was quantified by BAS1800 Bioimaging analyzer.

FIG. 1. PTC4 overexpression suppresses the lethality of the Δcnb1 Δmpk1 strain by negatively regulating Hog1 MAPK. A, effect of Hog1 deletion (Δhog1) on the growth of Δcnb1 Δmpk1 strain. WT (W303), Hog1 (SOSS), Δcnb1 (DTT14), Δmpk1 (TNF46), Δcnb1 Δmpk1 (TNF36), or Δhog1 Δcnb1 Δmpk1 (SOS48) cells were spotted on agar plates of YPD, or YPD + 1 M sorbitol (+Sor), and cultured for 2 days at the indicated temperature. B, effect of PTC4 overexpression on the high osmolarity-induced Hog1 phosphorylation. WT (W303) cells transformed with YEp51 (vector) or YEp51-PTC4 (pGAL-PTC4) were grown in SR minus leucine (same composition as SD minus leucine, except 2% raffinose was used instead of dextrose) to early log phase and shifted to 30 °C (WT) or to 37 °C (vector). Whole-cell extracts were prepared, and immunoblotting was performed with anti-phospho-p38 or anti-HA as a loading control. C, Hog1 kinase activity was measured using the same cell extracts. Immunoprecipitated Hog1-HA was incubated with MBP and γ-[32P]ATP. The level of Hog1-HA in lysate was assessed by immunoblotting with anti-HA antibody. The radiolabel incorporated into MBP was quantified by BAS1800 Bioimaging analyzer.

might have been the result of the down-regulation of the HOG pathway by PTC4 overexpression. If this were the case, the lethality of the Δcnb1 Δmpk1 strain should be suppressed by a defective Hog pathway. As expected, the Δcnb1 Δmpk1 Hog1 triple deletion mutant was viable on YPD plates at 30 °C and even at 37 °C (Fig. 1A). In contrast, the Δcnb1 Δmpk1 double deletion had no significant effect on the osmosensitivity of the Δhog1 strain (Fig. 1A), suggesting that the Ca$^{2+}$ signaling pathway and the HOG pathway are antagonistic in the growth regulation, but not in the high osmolarity response. Hog1 activation is the result of the phosphorylation of both Thr-174 and Tyr-176. To examine whether the PTC4 gene down-regulates Hog1, we compared the levels of Hog1 phosphorylation in the strains with or without overexpressing.

Fig. 1. PTC4 overexpression suppresses the lethality of the Δcnb1 Δmpk1 strain by negatively regulating Hog1 MAPK. A, effect of Hog1 deletion (Δhog1) on the growth of Δcnb1 Δmpk1 strain. WT (W303), Hog1 (SOSS), Δcnb1 (DTT14), Δmpk1 (TNF46), Δcnb1 Δmpk1 (TNF36), or Δhog1 Δcnb1 Δmpk1 (SOS48) cells were spotted on agar plates of YPD, or YPD + 1 M sorbitol (+Sor), and cultured for 2 days at the indicated temperature. B, effect of PTC4 overexpression on the high osmolarity-induced Hog1 phosphorylation. WT (W303) cells transformed with YEp51 (vector) or YEp51-PTC4 (pGAL-PTC4) were grown in SR minus leucine (same composition as SD minus leucine, except 2% raffinose was used instead of dextrose) to early log phase and shifted to 30 °C (WT) or to 37 °C (vector). Whole-cell extracts were prepared, and immunoblotting was performed with anti-phospho-p38 or anti-HA as a loading control. C, Hog1 kinase activity was measured using the same cell extracts. Immunoprecipitated Hog1-HA was incubated with MBP and γ-[32P]ATP. The level of Hog1-HA in lysate was assessed by immunoblotting with anti-HA antibody. The radiolabel incorporated into MBP was quantified by BAS1800 Bioimaging analyzer.
the Hog1 kinase activity in the Δcnb1 Δmpk1 strain. As the result, overexpression of these genes reduced the basal level activity of Hog1 kinase in the Δcnb1 Δmpk1 strain (Fig. 2B). The PTP2 overexpression inhibited the Hog1 activity, but it could not suppress the growth defect (Fig. 2B). For the difference in the effect of the overexpression of these genes on the growth of the Δcnb1 Δmpk1 strain, see “Discussion.”

Δhog1 Mutant Displays Ca2+-induced G2 Delay and Polarized Bud Growth—Deletion of the HOG pathway has been shown to partially suppress the defect of the mutants in cell wall integrity signaling in a manner mediated by the SVG (STE vegetative growth) pathway that regulates the cell wall integrity in parallel with the Mpk1 MAPK pathway (27). In this process, the HOG pathway seemed to negatively regulate cell wall integrity through the SVG pathway, antagonizing the Mpk1 MAPK pathway. Supporting this possibility, it was shown that the defect of the HOG pathway causes an activation of the STE pathway (28), which in turn may lead to the activation of cell wall integrity.

The HOG pathway seems to be also involved in G2/M cell-cycle regulation. Deletion of HSL1 gene, which encodes a negative regulator of Swe1 kinase, suppressed the lethality caused by the double deletions of the CNB1 and MPK1 genes, suggesting that the HOG pathway and the Ca2+ signaling pathways also antagonize in G2/M cell-cycle progression (data not shown). In the Ca2+-triggered, Swe1-mediated G2/M cell-cycle regulatory mechanism, Zds1 inhibits SWE1 transcription in G2 phase, antagonizing the Ca2+ signaling pathways (21, 29). Therefore, the Δzds1 strain exhibits various Ca2+ phenotypes, including hypersensitivity to CaCl2 (>50 mM), G2 cell-cycle delay, and polarized bud growth. If the HOG pathway and the Ca2+ signaling pathways antagonize one another in cell-cycle regulation, it would be expected that the Δhog1 strain growing in the presence of CaCl2 would display a phenotype analogous to that of the Δzds1 strain. This was indeed the case. The Δhog1 strain exhibited the Δzds1 phenotypes similar to those of the Δcnb1 phenotype (Fig. 3, A and B). The similar phenotypes were not observed with the Δhog1 strain in the presence of CaCl2 or other cations (e.g. Mg2+ and Na+), suggesting that the Ca2+ phenotypes exhibited by the Δhog1 strain were distinct from those of the high osmolarity of the solutes (Fig. 3A and data not shown).

The Ca2+ phenotypes of the Δzds1 strain were shown earlier to be suppressed by the deletion of the CNB1, MPK1, or SWE1 gene (21). So we asked whether calcineurin, Mpk1, or Swe1 is required for the phenotype displayed by the Δhog1 strain. The effects of CaCl2 on the Δhog1 strain were all rescued by the deletion of CNB1 or SWE1, but not that of MPK1 (Fig. 3, A and B). However, because the deletion of the SWE1 gene was only partial, it was suggested that the HOG pathway has additional target(s) in the Ca2+-mediated growth regulation.

Swe1 Degradation in the Δhog1 Strain Is Delayed by Ca2+—

The ΔSWE1 mRNA level of WT cells fluctuates during the cell cycle, peaking in G2/S and declining just before the onset of mitosis (21, 29). We first examined whether the HOG pathway is involved in the transcriptional regulation of the ΔSWE1 gene by measuring the ΔSWE1 mRNA levels in the presence or absence of 50 mM CaCl2 in a synchronized cell cultures by a-factor. The fluctuation pattern was not dependent on the presence of the HOG1 gene, indicating that Hog1 is not important for the transcriptional regulation of the ΔSWE1 gene (data not shown).

We next examined whether the HOG pathway is involved in post-transcriptional Swe1 regulation. The accumulation of Swe1 is periodic during the cell cycle, peaking at the time of bud emergence and declining before nuclear division (30, 31). We constructed various strains (WT, Δcnb1, Δhog1, and Δhog1 Δcnb1, each on the Δswe1 background) expressing a Myc

PTC4 gene from the GAL1 promoter by using polyclonal antibody against phospho-p38, which cross-reacts with phospho-Hog1. As expected, the level of the high osmolarity-induced Hog1 phosphorylation was significantly reduced by PTC4 overexpression (Fig. 1B). This result was further verified by measuring the Hog1 kinase activity using [γ32P]ATP and MBP as a substrate. Indeed, the kinase activity was reduced by PTC4 overexpression (Fig. 1C). To see whether the suppression of this lethality is the result of the inhibition of the HOG pathway, we examined whether the overexpression of other negative regulators (PTC1, -2, -3, and -PTP2) known for the HOG pathway can suppress the lethality of the Δcnb1 Δmpk1 strain. Overexpression of the PTC1, -2, or -3 gene could suppress the lethality to a degree similar to the overexpression of the PTC4 gene (Fig. 2A). However, overexpression of the PTP2 gene failed to suppress the lethality (Fig. 2A). We next examined whether overexpression of PTC4 or PTP2 gene truly reduced the Hog1 kinase activity in the Δcnb1 Δmpk1 strain.
epitope-tagged Swe1 from the chromosomally integrated gene and compared the fluctuation patterns of Myc-tagged Swe1 by immunoblot analysis in synchronized cell cultures. The Myc-Swe1 construct was fully functional in vivo (data not shown).

The G1 cells synchronized with \( /H9251\)-factor were released into YPD medium with or without 50 mM CaCl\(_2\). The cell-cycle progression in synchronous cell cultures was monitored by the determination of nuclear division and DNA contents (Fig. 4, B and C). As previously reported (30, 31), the Swe1 level of the WT strain fluctuated during the cell cycle. The slower migrating species of Swe1 represent a phosphorylated isoform, and the phosphorylation occurs prior to its degradation coincidental with the onset of mitosis (31). Immediately after the degradation, the faster migrating species of Swe1 re-appeared. In YPD medium, there was no significant difference among the strains in the periodic patterns of the Swe1 level and the timing of nuclear division. However, in the \( /H9004\) cells cultivated with CaCl\(_2\), the Swe1 level was sustained for 30 min longer, and correspondingly, the onset of mitosis was delayed by 30 min, in comparison with those in WT cells treated similarly. This result suggests that Ca\(^{2+}\) caused a delay in Swe1 degradation in the \( /H9004\) strain. Both Ca\(^{2+}\)-induced Swe1 stabilization and the delay of nuclear division in the \( /H9004\) strain were shortened to 15 min by \( /H9004\) deletion, indicating that the Swe1 stability is antagonistically regulated in a manner mediated by the HOG pathway and calcineurin (compare \( /H9004\) and \( /H9004\) strains in Fig. 4A).

The HOG Pathway Promotes Bud Emergence, whereas Calcineurin Inhibits Actin Polarization—Using the same synchronous cell cultures described in the previous section, we also determined the time course of bud emergence (Fig. 4B). In YPD medium, no significant difference was observed among the strains in the timing of bud emergence. In the presence of 50 mM CaCl\(_2\), bud emergence in the \( /H9004\) strain was delayed by 30 min compared with that in the WT strain. In the presence of sorbitol, the \( /H9004\) strain showed a delay of bud emergence by 15 min compared with that of the WT strain. The Ca\(^{2+}\)-induced delay in the \( /H9004\) strain was shortened to 15 min (compare...
Fig. 4. Effects of CaCl₂ on the Swe1 accumulation, nuclear division, bud formation, and DNA content in synchronized cell culture of various strains. A, the fluctuation of the Swe1 abundance during the cell cycle in the cells synchronized with α-factor. WT (SHI26), Δcnb1 (SHI63), Δhog1 (SHI27), or Δhog1 Δcnb1 (SHI30) strains were grown in YPD to early-log phase, and the cells were then synchronized as described under “Materials and Methods.” Whole-cell extracts were prepared and analyzed by immunoblotting with an antibody against Myc-Swe1 and one against Cdc28 as a loading control.

B, quantification of cumulative percentage of nuclear division and bud formation during cell-cycle progression in various strains in the cell cultures prepared as in A was determined by microscopic observation following PI staining; at least 300 cells were counted for each time point. The cells were released into YPD medium with or without 50 mM CaCl₂ (+Ca²⁺) or 150 mM sorbitol (+Sor). Samples were taken at an interval of 15 min after removing the α-factor.

C, DNA content of various strains in a synchronized culture prepared as in A after release from arrest in G₁.
\( \Delta hog1 \) and \( \Delta hog1 \Delta cnb1 \) in Fig. 4B). In contrast, the sorbitol-induced delay was not altered in the \( \Delta cnb1 \) strain (Fig. 4B). Moreover, no significant delay was observed in DNA replication of various strains in the presence of 50 mM CaCl\(_2\), indicating that the Ca\(^{2+}\)-induced delay of bud formation is not simply a reflection of the decelerated cell cycle by Ca\(^{2+}\) (Fig. 4C). Taken together, these results indicated that bud emergence is antagonistically regulated, positively by the HOG pathway and negatively by calcineurin.

To address whether Ca\(^{2+}\), rather than the osmotic stress, is specifically responsible for the severe delay in mitosis caused by CaCl\(_2\), we examined whether osmotic stress of sorbitol might induce a similar effect. In YPD medium containing 150 mM sorbitol, which gives an osmolality equivalent to that of 50 mM CaCl\(_2\), both Swe1 degradation and the nuclear division of the \( \Delta hog1 \) cells were delayed by 15 min compared with those of WT cells. This delay was shorter than that caused by CaCl\(_2\) (30 min; Fig. 4, A and B). Moreover, the delays in Swe1 degradation and cell-cycle progression caused by sorbitol were not alleviated by the deletion of calcineurin, suggesting that the mechanisms underlying the delays were different for Ca\(^{2+}\) and sorbitol (Fig. 4, A and B).

To further clarify the roles of these pathways in the control of bud emergence, we examined the effects of CaCl\(_2\) on bud emergence and actin polarization at the bud site in \( \alpha \)-factor-synchronized cells (Fig. 5). Actin polarization was determined by rhodamine-phalloidin staining. No significant difference was observed in the kinetics of actin polarization among the strains (WT, \( \Delta cnb1 \), \( \Delta hog1 \), and \( \Delta hog1 \Delta cnb1 \)) after release of the cells to YPD medium. The timing of actin polarization of WT and the \( \Delta hog1 \) cells was only slightly delayed after release to the medium containing 50 mM CaCl\(_2\) (compare +Ca\(^{2+}\) at 15 min for WT and \( \Delta hog1 \) cells). However, bud emergence of the \( \Delta hog1 \) cells was delayed from that of WT cells by 30 min (compare WT and \( \Delta hog1 \) cells, +Ca\(^{2+}\) at 45–60 min), although the actin patches were localized apparently normally at the presumptive bud site. The delay in actin polarization was nearly completely abolished in the \( \Delta cnb1 \) cells, although bud emergence was still delayed by 15 min (compare WT and \( \Delta hog1 \) cells, +Ca\(^{2+}\) at 15–30 min). The Ca\(^{2+}\)-induced delay of bud emergence in the \( \Delta hog1 \) strain was only partially suppressed by the deletion of calcineurin (compare \( \Delta hog1 \) and \( \Delta hog1 \Delta cnb1 \) cells, +Ca\(^{2+}\) at 45 min).

Calcineurin is known to regulate cellular Ca\(^{2+}\) homeostasis. Defect of calcineurin leads to increase Ca\(^{2+}\) tolerance by increasing vacular sequestration of Ca\(^{2+}\) from the cytosol (32, 33). Therefore, the effect of \( \Delta cnb1 \) on actin polarization could be the result of an alteration of cellular Ca\(^{2+}\) homeostasis. We investigated whether calcineurin truly regulates actin polarization by using a constitutively active form of calcineurin, containing the C-terminal truncated form of catalytic subunit (CMP24C). The galactose-inducible GAL1-CMP24C construct was introduced to the URA3 locus of WT strain and an experiment similar to that described above was performed without addition of CaCl\(_2\). Overexpression of CMP24C inhibited actin polarization to the bud site (Fig. 6, compare +glucose (off) and +galactose (on)). The overexpression of CMP24C did not lead to a delay in the progression of DNA replication (Fig. 6B) consistently with the results obtained with the addition of Ca\(^{2+}\) (Fig. 4C). These results indicated that the HOG pathway promotes bud emergence after actin polarization, whereas calcineurin inhibits actin polarization (see “Discussion”).

The Calcineurin-Crz1/Tcn1 Pathway Is Required for the Down-regulation of the HOG Pathway—To further investigate the antagonizing mechanism between the 2 pathways, we examined whether calcineurin down-regulates the HOG pathway by determination of phospho-Hog1 with anti-phospho-pS8 antibody. In WT cells, the Hog1 phosphorylation induced by CaCl\(_2\) was transient, peaking at 15 min and disappearing by 30 min of the treatments (Fig. 7A). In the \( \Delta cnb1 \) strain, Hog1 phosphorylation was induced by CaCl\(_2\) similarly as in WT strain, but the phosphorylation level increased to a much higher level and sustained longer than in the WT one (Fig. 7A). We further examined whether Crz1/Tcn1, a transcription factor activated by calcineurin, was involved in this process (17, 18). The level of Ca\(^{2+}\)-induced Hog1 phosphorylation in the \( \Delta crz1 \) strain was similarly high as that in the \( \Delta cnb1 \) strain (Fig. 7A). Moreover, \( \Delta crz1 \) and \( \Delta cnb1 \) mutations did not exhibit an additive effect on the level of Hog1 phosphorylation, suggesting that calcineurin and Crz1 regulate Hog1 phosphorylation in a common pathway (data not shown). The Ca\(^{2+}\)-induced Hog1 hyperphosphorylation in the \( \Delta crz1 \) strain led to an increased expression level of GDP1 mRNA compared with that in the WT strain, suggesting that the negative regulation of the HOG pathway was mediated by the Crz1 transcription factor (data not shown).

DISCUSSION

HOG Pathway and Calcineurin Antagonize in Growth Regulation—We have shown here that the Hog1 deletion remarkably suppressed the lethality caused by the simultaneous loss of calcineurin and the Mpk1 MAPK pathway. Thus, the \( \Delta cnb1 \) \( \Delta mpk1 \) \( \Delta hog1 \) triple disruption mutant grew apparently normally on YPD plate at 30 °C and even at 37 °C, a restrictive temperature for the \( \Delta mpk1 \) strain. The lethality of the \( \Delta cnb1 \) \( \Delta mpk1 \) strain is attributable mainly to a defect in the cell wall construction for the following reasons. 1) The lethality is rem-
Calcineurin negatively regulates actin polarization and bud emergence, whereas Hog1 positively regulates bud formation after actin polarization. WT (W303), Δcnb1 (DHT14), Δhog1 (SOS5), or Δhog1 Δcnb1 (SOS31), Δptp2 (SH7687), or Δptp2 Δcnb1 (SH334) was grown in YPD medium to early-log phase, and cells were synchronized as described under “Materials and Methods.” The cells were released into
ed by osmotic stabilizers added to the medium (20). 2) The expression of the FKS2 gene, which encodes a glucan synthetase, is activated by the calcineurin-Crz1 pathway (35). 3) The Mpk1 MAPK cascade has been implicated in the expression of various genes involved in cell wall synthesis (1). The observation that the triple disruption mutant was viable on YPD plates even at 37 °C indicates that the HOG pathway negatively regulates the cell wall construction, antagonizing the Ca^{2+} signaling pathways.

Besides the cell wall, calcineurin and the Mpk1 pathway coordinately regulate the G_{1}/M cell-cycle progression through the regulation of Swe1 (21). Because the suppression of the synthetic lethality by the HOG1 deletion was very potent, the HOG pathway and the Ca^{2+} signaling pathways were thought to counteract each other in diverse events of growth regulation, including cell wall synthesis and cell-cycle progression. In fact, the synthetic lethality was partially suppressed by the deletion of the Nim1-like kinase Hsl1, the negative regulatory kinase of Swe1 (data not shown). The involvement of the HOG pathway in cell-cycle regulation was further supported by the observations that the HOG pathway regulates stability of Swe1 (Fig. 3A) and the effect of HOG1 deletion was partially suppressed by the SWEL deletion (data not shown).

The \( \Delta \text{cnb1} \Delta \text{mpk1} \) synthetic lethality was suppressed by overexpression of \( \text{PTC1} \), \(-2\), \(-3\), or \(-4\), encoding the serine/threonine phosphatase PP2C family known as the negative regulator of the HOG pathway, supporting the notion that the HOG pathway and the Ca^{2+} signaling pathways antagonize each other in growth regulation. However, overexpression of the tyrosine phosphatase Ptp2 failed to suppress the lethality. This result suggests that the PP2C overexpression suppressed the growth of the \( \Delta \text{cnb1} \Delta \text{mpk1} \) strain by dephosphorylating a target protein phosphorylated by the serine/threonine kinase Hog1. Further investigation is necessary to elucidate the mechanism of the lethality.

Calcineurin and the HOG Pathway Regulate Distinct Processes of Bud Emergence—The HOG pathway and calcineurin/ Mpk1 MAPK pathways perform antagonistic roles in the regulation of bud emergence. The detailed analysis of the kinetics of the bud emergence and the actin localization in synchronized cell cultures revealed that calcineurin and the HOG pathway regulate distinct processes of bud emergence (Fig. 5). In the WT strain, the actin polarization at the presumptive bud site and bud emergence were shortly delayed (15 min) by Ca^{2+}. The delays were abrogated in the \( \Delta \text{cnb1} \) strain, indicating that the delays occurred in a calcineurin-dependent manner. Compared with the wild-type strain, the delay in bud emergence induced by Ca^{2+} in the \( \Delta \text{hog1} \) strain was longer. Although bud emergence in the \( \Delta \text{hog1} \) strain was severely delayed by Ca^{2+}, actin polarization was only slightly affected. These results indicated that the Hog1 activation stimulated bud emergence at a step after actin had correctly become localized at the bud site. The Ca^{2+}-induced delay of bud emergence in the \( \Delta \text{hog1} \) strain was only partially suppressed by the deletion of calcineurin, suggesting that the \( \text{CNB1} \) deletion can suppress the defect in actin polarization, but not the bud emergence. Taken together, these results indicated that the processes of the bud emergence that are regulated by calcineurin and the HOG pathway are distinct. Calcineurin inhibits actin polarization, an early step of bud emergence, whereas Hog1 promotes bud emergence at a step after actin has become polarized. The identification of the targets of calcineurin in regulating actin polarization and that of the HOG pathway in bud emergence still remains to be made.

The G_{2} delay caused by 50 mM CaCl_{2} in the \( \Delta \text{hog1} \) strain may have been the result of the activation of the morphogenesis checkpoint. Supporting this notion, bud formation in the \( \Delta \text{hog1} \) strain was severely delayed, although DNA replication was not significantly affected by CaCl_{2} (Fig. 4, B and C). The morphogenesis checkpoint monitors abnormal bud formation and is functional in late G_{1} to S phases, but is nonfunctional once the bud has grown to a critical size (31). It is possible that the defect of bud emergence caused by deregulated synthesis of the cell wall and the inhibition of the actin polarization in the \( \Delta \text{hog1} \) may activate the morphogenesis checkpoint. Why did the \( \Delta \text{hog1} \) strain exhibit a severe defect in bud emergence in the presence of Ca^{2+}? As a yeast cell buds, the cell wall must expand to fit the expanding plasma membrane. During expansion of the rigid cell wall, it must be weakened at the point of new growth to add newly made cell wall material. The severe defect in bud emergence in the \( \Delta \text{hog1} \) cells would be explained by the deregulated synthesis of the cell wall at the bud site by the hyperactivation of the cell wall synthesis as a result of the absence of its negative regulator (Hog1) and by the activation of the Ca^{2+} signaling pathways. Alternatively, it is also possible that the delay in bud emergence might have resulted from the...
defect in recovery from the inhibition of protein synthesis. It was recently reported that the \( \text{H}9004 \) \( \text{hog1} \) mutant was defective in the recovery from hypertonic stress-induced inhibition of protein synthesis (36).

It was suggested that Ca\(^{2+}\) signal inhibited bud emergence through inhibition of Hog1 in a manner mediated by the calcineurin-Crz1 pathway and through inhibition of actin polarization by calcineurin. If so, the inhibitory effect of Ca\(^{2+}\) on bud emergence would be abolished by the activation of Hog1. This was indeed the case. The delay in bud emergence that was still seen in the \( \text{H}9004 \) \( \text{cnb1} \) strain was apparently totally abolished by the deletion of the \( \text{PTP2} \) gene (Fig. 5B).

We showed here that calcineurin was involved in the inhibition of actin polarization at the bud site. In the pathogenic fungus \( \text{Cryptococcus neoformans} \), it was earlier suggested that calcineurin was required for the polar cell growth in mating and haploid fruiting (37). In the nerve cells, calcineurin was implicated in cell polarization (38).

The Ca\(^{2+}\)-induced inhibition of actin polarization in \( \Delta \text{hog1} \) strain was not suppressed by the deletion of \( \text{CRZ1} \) (data not shown), suggesting that other target molecule(s) are involved in the regulation of actin polarization by calcineurin.

Cross-talk between the HOG Pathway and Ca\(^{2+}\) Signaling Pathway—Because the HOG pathway and the Ca\(^{2+}\) signaling pathways are regulated opposingly by high and low external osmolarity, respectively, the question arises as to whether these pathways regulate each other. Our data suggested that the calcineurin-Crz1 pathway was involved in the down-regulation of the HOG pathway through the regulation of the Sln1, but not Sho1, branch (Fig. 7A; see a model in Fig. 8). Further, Hog1 phosphorylation was sustained with the \( \Delta \text{ste11} \) \( \Delta \text{cnb1} \) strain, but not with the \( \Delta \text{ssk1} \) \( \Delta \text{cnb1} \) strain, suggesting that the calcineurin-Crz1 pathway is involved in the inhibition of the Sln1 pathway. The phosphorylation level of Hog1 in the \( \Delta \text{ssk1} \) \( \Delta \text{cnb1} \) strain decreased after 45 min of its activation, suggesting that the down-regulation of the Sln1 branch by the cal-
calcineurin-Crz1 pathway is important in the cross-talk regulation of the HOG pathway (Fig. 7A). The Hog1 phosphorylation in the ∆cnb1 strain induced by 100 mM Ca²⁺ was significantly higher and sustained longer than that by 300 mM sorbitol, suggesting that Ca²⁺ is more potent than sorbitol in Hog1 activation (compare the phosphorylation levels of “∆cnb1” samples in Fig. 7, A and B). Because hyperosmotic stress induces a transient increase in cytosolic Ca²⁺ from vacuolar stores (34), the difference in the effect of CaCl₂ and sorbitol on Hog1 phosphorylation may simply reflect the difference in the effect of exogenous CaCl₂ and hyperosmotic stress on the elevation of intracellular Ca²⁺. How does Ca²⁺ activate the Snl1 branch of the HOG pathway? As previously suggested, the osmo-activated Snl1-Ypd1-Ssk1 phosphorylase system may be under a negative feedback regulation by accepting a phosphate group from glycerol 3-phosphate or related metabolic intermediate donors, which may be overproduced by glycerol-3-phosphate dehydrogenase, a target of the HOG pathway (5). The loss of calcineurin activity leads to elevation of the cytosolic Ca²⁺ concentration (32, 39, 40). If such a feedback regulatory mechanism that is mediated by a phosphorylated metabolite does exist for the HOG pathway, the elevation of intracellular Ca²⁺ may cause the formation of an insoluble complex with the phosphate compound, interfering with re-phosphorylation of the two-component system. Alternatively, it is possible that phosphate compound, interfering with re-phosphorylation of the p38 family MAPK pathway by the Ca²⁺ pathway and the HOG pathway. The activation of the p38 MAPK cascade by Ca²⁺ signal was recently reported with the p38 MAPK cascade of Caenorhabditis elegans (41). In mammalian cells, the p38 MAPK pathway is inhibited by calcineurin in a manner mediated by the transcriptional up-regulation of MAPK phosphatase-1 (42). It is possible that regulation of the p38 family MAPK pathway by the Ca²⁺ signaling pathways is conserved from yeast to higher eukaryotes.

On the basis of these data, we propose a model for the regulation of cell growth by the HOG pathway and calcineurin (Fig. 8).

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