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Regulation of Cell Cycle Progression by Swe1p and Hog1p Following Hypertonic Stress

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Exposure of yeast cells to an increase in external osmolarity induces a temporary growth arrest. Recovery from this stress is mediated by the accumulation of intracellular glycerol and the transcription of several stress response genes. Increased external osmolarity causes a transient accumulation of 1N and 2N cells and a concomitant depletion of S phase cells. Hypertonic stress triggers a cell cycle delay in G2 phase cells that appears distinct from the morphogenesis checkpoint, which operates in early S phase cells. Hypertonic stress causes a decrease in *CLB2* mRNA, phosphorylation of Cdc28p, and inhibition of Clb2p-Cdc28p kinase activity, whereas Clb2 protein levels are unaffected. Like the morphogenesis checkpoint, the osmotic stress-induced G2 delay is dependent upon the kinase Swe1p, but is not tightly correlated with inhibition of Clb2p-Cdc28p kinase activity. Thus, deletion of *SWE1* does not prevent the hypertonic stress-induced inhibition of Clb2p-Cdc28p kinase activity. Mutation of the Swe1p phosphorylation site on Cdc28p (Y19) does not fully eliminate the Swe1p-dependent cell cycle delay, suggesting that Swe1p may have functions independent of Cdc28p phosphorylation. Conversely, deletion of the mitogen-activated protein kinase *HOG1* does prevent Clb2p-Cdc28p inhibition by hypertonic stress, but does not block Cdc28p phosphorylation or alleviate the cell cycle delay. However, Hog1p does contribute to proper nuclear segregation after hypertonic stress in cells that lack Swe1p. These results suggest a hypertonic stress-induced cell cycle delay in G2 phase that is mediated in a novel way by Swe1p in cooperation with Hog1p.

INTRODUCTION

The cell cycle is the orderly progression of events that allows a cell to replicate and segregate its genome. In yeast, progression of the cell cycle is driven by a single cyclin-dependent kinase, called Cdc2 in fission yeast and Cdc28p in budding yeast (reviewed in Hayles and Nurse, 1989; Nasmyth, 1993; Lew *et al.*, 1997). Cdc28p and Cdc2 trigger cell cycle phase-specific events by differential association with obligatory-activating subunits called cyclins. During the cell cycle, cyclin levels are regulated by a complex system of transcriptional regulation and proteolysis (Deshaies, 1995; Nasmyth, 1996). In *Saccharomyces cerevisiae,* Cdc28p is activated in late G1 phase by the G1 cyclins Cln1p and Cln2p, and in G2/M phase by the B-type cyclins Clb1p and Clb2p.

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Cell cycle progression is regulated by checkpoint mechanisms that monitor critical processes and delay the cell cycle to allow error-free completion of such processes before later cell cycle events are initiated. Some examples of checkpoint targets are DNA damage (Weinert and Hartwell, 1988), DNA replication (Weinert *et al.*, 1994), kinetochore attachment to the mitotic spindle (Rudner and Murray, 1996), and bud morphogenesis (Lew and Reed, 1995). Inhibition of cyclin-dependent kinase activity (Cdc2 or Cdc28p) is the means by which some but not all checkpoint pathways enforce a delay in cell cycle progression. As an example, in fission yeast, DNA damage and defective DNA replication halt entry into mitosis by triggering the Wee1/Mik1-dependent phosphorylation of Cdc2 on tyrosine 15, which inhibits the kinase activity (Gould and Nurse, 1989; Lundgren *et al.*, 1991; Rhind *et al.*, 1997; Rhind and Russell, 1998). Cells that lack Wee1 are thus sensitive to DNA damage. Tyrosine phosphorylation of Cdc2 plays an important role in the normal progression of the cell cycle because wee1 mutants do not sense nutrient conditions properly and proceed through mitosis prematurely (reviewed in MacNeill and Nurse, 1997). Conversely, cells that lack Cdc25, a tyrosine

Table 1. Yeast strains

W303	MAT**a** *ura3-1 ade2-1 trp1-1 his3-11 leu2-3,112 can1-100*
MT588	MAT**a** *CLB2-3XHA*
MT588 *swe1*Δ	MAT**a** *CLB2-3XHA swe1*Δ::*LEU2*
MT588 *hog1*Δ	MAT**a** *CLB2-3XHA hog1*Δ::*TRP1*
MT588 *gpd1*Δ *gpd2*Δ	MAT**a** *CLB2-3XHA gpd1*Δ::*LEU2 gpd2*Δ::*URA3*
MT588 *swe1*Δ *gpd1*Δ *gpd2*Δ	MAT**a** *CLB2-3XHA swe1*Δ::*LEU2 gpd1*Δ::*TRP1 gpd2*Δ::*URA3*
MT588 *hog1*Δ *swe1*Δ	MAT**a** *CLB2-3XHA hog1*Δ::*TRP1 swe1*Δ *LEU2*
MT588 *hog1*Δ *gpd1*Δ *gpd2*Δ	MAT**a** *CLB2-3XHA hog1*Δ::*TRP1 gpd1*Δ::*LEU2 gpd2*Δ::*URA3*
CDC28-HA	MAT**a** *CDC28-HA*
CDC28-HA^{Y19F}	MAT**a** *CDC28-HA^{YF}*

phosphatase that removes the inhibitory phosphate from Y15 of Cdc2, arrest in G2 phase (Russell and Nurse, 1986). Regulation of Cdc25 phosphatase activity (Rhind *et al.*, 1997) and regulation of Cdc25 localization (Lopez-Girona *et al.*, 1999) are additional components of the response to DNA damage that affect Cdc2 phosphorylation.

Budding yeast contains an analogous regulatory circuit based on phosphorylation of the corresponding tyrosine (Y19) on Cdc28p by the Swe1p kinase, and removal of the inhibitory phosphate by the Mih1p phosphatase (Russell *et al.*, 1989; Booher *et al.*, 1993). However, deletion of *SWE1* or a Y19F point mutation in Cdc28p has no obvious effect on growth or viability in *S. cerevisiae* (Sorger and Murray, 1992; Booher *et al.*, 1993). Despite this, Cdc28p phosphorylation has been identified as a component of the bud morphogenesis checkpoint (Lew and Reed, 1995). Activation of this checkpoint by a *cdc24-1* block in bud formation stimulates Swe1p-dependent tyrosine phosphorylation of Cdc28p, inhibition of Clb2p and Clb3p associated Cdc28p kinase activity, a delay in the accumulation of *CLB2* mRNA and a consequent delay in G2 phase (Sia *et al.*, 1996). Recent studies indicate that the morphogenesis checkpoint responds to disruption of the actin cytoskeleton (McMillan *et al.*, 1998) and/or disruption of septin structures required for cytokinesis (Barral *et al.*, 1999).

Yeast cells are normally exposed to a variety of environmental stresses. Some of these stresses, for example, oxidative stress (Lee *et al.*, 1996; Wanke *et al.*, 1999) and mild heat shock (Rowley *et al.*, 1993; Raboy *et al.*, 1999), cause arrest of the cell cycle in G1. Increases in extracellular osmolarity also induce a variety of cellular responses. Many of these responses are mediated by the HOG pathway (Banuett, 1998; Gustin *et al.*, 1998) in one of five mitogen-activated protein (MAP) kinase signaling pathways in *S. cerevisiae*. Increasing the external osmolarity induces expression of osmoregulatory genes such as *GPD1* and *HOR2/GPP2* and stress response genes such as *CTT1* and *HSP12*. Deletion of HOG pathway genes, such as the MAP kinase Hog1p, specifically blocks induction of these osmoregulation and stress response genes by osmotic stress, but has little or no effect on regulation of these genes by other stresses (Albertyn *et al.*, 1994a; Schuller *et al.*, 1994; Hirayama *et al.*, 1995; Varela *et al.*, 1995; Norbeck *et al.*, 1996). Osmotic stress activation of Hog1p, measured as increases in Hog1p phosphorylation (Brewster *et al.*, 1993) or Hog1p movement into the nucleus (Ferrigno *et al.*, 1998; Reiser *et al.*, 1999), occurs within minutes after increasing the osmolarity, correlating with the activation of gene expression. HOG pathway mutants have a complex phenotype, which includes cell morphological defects that suggest a lack of coordination between the cell cycle and cell growth (Brewster and Gustin, 1994). Not all responses to osmotic stress involve the HOG pathway. For example, the osmotic stress induced loss of actin cytoskeleton organization (Chowdhury *et al.*, 1992) is unaffected by HOG pathway mutations (Brewster and Gustin, 1994).

Although previous work has suggested that hypertonic shock early in the cell cycle can affect Swe1p stability (Sia *et al.*, 1998), the effect of hypertonic shock on cell cycle components and cell cycle progression has not been thoroughly investigated. In this report, we show that an increase in extracellular osmolarity also triggers a G2 delay that is similar to, but distinct from the morphogenesis checkpoint. The delay caused by hypertonic shock involves changes in Cdc28p phosphorylation and changes in Cdc28p enzymatic activity that are dependent on Swe1p and the MAP kinase Hog1p, respectively. These observations suggest a complex interplay between the Swe1p and Hog1p pathways underlies the cell cycle response to hypertonic stress.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

Cells were grown in YEP medium (1% yeast extract, 2% peptone) supplemented with either 2% dextrose or 2% raffinose where indicated. Galactose induction was accomplished by initial growth in YEP + raffinose, followed by addition of galactose to 2%. Strains used are listed in Table 1 and were derivatives of W303. The strain used as a positive control for the antiphospho-Cdc2 antibody was created by deleting *MIH1* in W303 as described in Booher *et al.* (1993). Wee1 under the control of a galactose-inducible promoter was subsequently integrated into the genome of the *mih1*Δ*::LEU2* strain as described in Russell *et al.* (1989).

Flow Cytometry, Cell Synchrony, and Determination of Mitotic Index

DNA content of cell cultures was determined as described in Tyers *et al.* (1993) by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Synchronized G1 cells were obtained from a 1.5-liter mid-log phase YEP + raffinose culture by using centrifugal elutriation as described in Tyers *et al.* (1993). Fractions that contained >95% unbudded cells were incubated at 30°C with shaking until ~80% budded at which point the culture was split in two and NaCl was added to 0.4 M to one half, whereas the other control culture was untreated. At each time point, aliquots were removed to determine the percentage of divided cells, the percentage of cells that had undergone nuclear division but not cytokinesis, and the mitotic

index (Lew and Reed, 1995). Mitotic index was determined as the percentage of cells with two nuclei plus the percentage of cells that had completed cell division.

$$\text{Mitotic Index} = \%\ \text{cells with two nuclei} + \%\ \text{cells that have divided}$$

$$\%\ \text{cells that have divided} = \frac{\text{no. cells}_t - \text{no. cells}_0}{\text{no. cells}_0} \quad (1)$$

Nuclei were visualized by staining the DNA with 4,6-diamidino-2-phenylindole. The number of cells that had completed cell division was calculated by determining the cell density at each time point, by using a hemacytometer. For each parameter, a minimum of 100 cells was counted at each time point.

For alpha factor experiments cells were grown in YEPD to an A_{600} of 0.3. Alpha mating factor was added at 24 μg/ml and the cultures incubated at room temperature for 2.5 h. The cultures were released from arrest by two washes with fresh media.

Analysis of mRNA, Protein, and Kinase Activity

Total RNA was isolated and analyzed as described (Cross and Tinkelenberg, 1991). Clb2p-associated Cdc28p kinase activity was determined by using an in vitro immunoprecipitation kinase assay on strains with an unmarked genomic copy of a triple hemagglutinin (HA) epitope-tagged Clb2p. Cell extracts were prepared and histone H1 kinase assays performed on Clb2p immunoprecipitates prepared from 1 mg of total cellular protein as described in Tyers *et al.* (1993). ^{32}P phosphorylated histone H1 was visualized by autoradiography and quantitated by using a MacBas Phosphorimager (Fuji, Stamford, CT) and a MacBas software package.

To analyze Cdc28p phosphorylation levels, cell extracts were prepared as described above and 1 mg of cell extract protein was incubated with p13^{suc1} agarose beads (Upstate Biotechnology, Lake Placid, NY). Alternatively, cell extracts were prepared as described above and HA-tagged Clb2p–Cdc28p complexes isolated by immunoprecipitation from 1 mg of cell extract protein by using the anti-HA 12CA5 monoclonal antibody plus protein A agarose beads. In each case, the beads were washed twice with lysis buffer, proteins extracted from the beads with SDS loading buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Immunoblots were probed with an antibody specific for phospho-Tyr15 of Cdc2 (New England Biolabs, Beverly, MA). Antibody cross-reactivity with phospho-Cdc28p was initially determined by using samples containing hyperphosphorylated Cdc28p protein extracts from a GAL-*wee1*$^+$ mih1Δ strain, and also samples containing nonphosphorylatable Cdc28p protein extracts from a *cdc28*^{Y19F} mutant. Antiphospho-Cdc2 antibodies showed significantly stronger specific immunoreactivity than anti-phosphotyrosine (our unpublished results). Antiphospho-Cdc2 antibody, anti-Cdc28p polyclonal antibody and 12CA5 monoclonal anti-HA antibody (Boehringer-Mannheim, Indianapolis, IN) were used at a 1:1000 dilution and detected with horseradish peroxidase secondary antibodies and enhanced chemiluminescence.

RESULTS

Hypertonic Shock Causes a Depletion of S Phase Cells

To determine the effect of mild osmotic stress conditions on cell cycle progression, flow cytometry was used to analyze the cellular DNA content of cultures that were exposed to 0.4 M NaCl (Figure 1A). We will subsequently refer to an increase in extracellular osmolarity as a hypertonic shock or stress. Within 30 min., addition of NaCl caused a reduction in the number of S phase cells with corresponding increases in the fraction of G1 and G2/M cells. Within 60 min, the S phase population had recovered and the histogram was very similar to that of an untreated control culture, indicating that the hypertonic stress response is transient. This result, along with an analysis of bud emergence and DNA content of synchronous cultures stressed in G1 (Figure 1B) suggested that hypertonic stress induces a cell cycle delay in both G2/M phase and G1 phase.

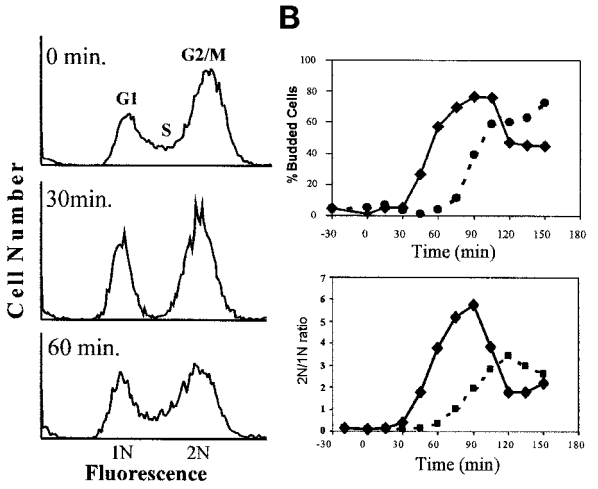


Figure 1. (A) Hypertonic stress results in a depletion of S phase cells from an asynchronous culture. Log phase cultures of wild-type MT588 were stressed by the addition of NaCl to a final concentration of 0.4 M. Samples were taken at the indicated times, and processed as described (see MATERIALS AND METHODS). The cellular DNA was stained with propidium iodide and analyzed by flow cytometry. Histograms were generated by using WinMDI software for the PC. Similar results were obtained in three separate experiments. (B) Hypertonic stress delays exit from G1. Wild-type MT588 were arrested in alpha factor, released, and stressed at time zero by the addition of 0.4 M NaCl. Budding index and ratio of 1N to 2N cells was determined for no salt control (solid line) and 0.4 M NaCl treated (dashed line) and plotted versus time in minutes after addition of NaCl.

Hypertonic Shock Causes a Decrease in CLB2 mRNA, and Inhibits Clb2p-Cdc28p Kinase Activity, but Does Not Affect Clb2p Levels

The mRNA levels of different cyclins were examined before and after hypertonic shock. Treatment with 0.4 M NaCl caused a rapid but temporary decrease in the level of *CLB2* mRNA (Figure 2A). Levels of other cyclin transcripts were also reduced, but not to the same degree as *CLB2* (our unpublished results). Because Clb2p-Cdc28p kinase activity stimulates *CLB2* transcription in a positive feedback loop (Amon *et al.*, 1993), the decrease in *CLB2* mRNA following hypertonic shock might be a consequence of reduced Clb2p-Cdc28p kinase activity. To test this idea, the Cdc28p kinase activity associated with wild-type levels of Clb2p was assayed in Clb2p immune complexes isolated from cells before and after exposure to hypertonic shock. These in vitro kinase assays used exogenously added histone H1 as a substrate. A decrease in Clb2p-Cdc28p kinase activity was observed fol-

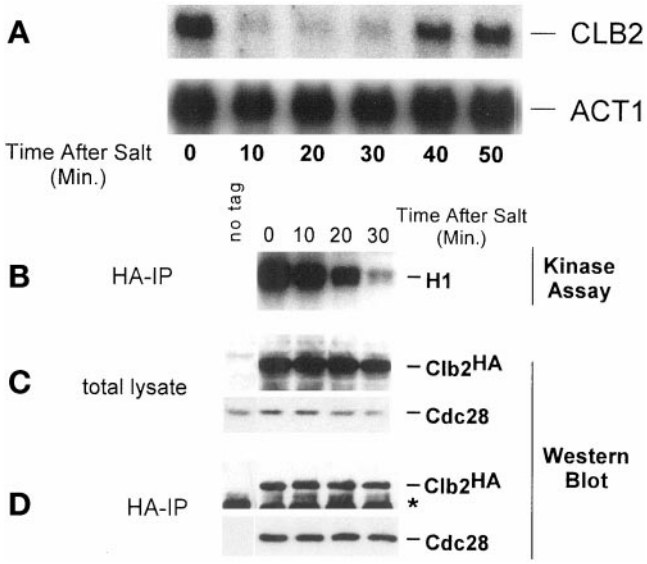


Figure 2. CLB2 mRNA levels and Clb2p-Cdc28p activity decrease in response to hypertonic shock. (A) Wild-type MT588 cells were grown to log phase and stressed by the addition of NaCl to 0.4 M. Samples were taken at the indicated times. Total RNA was isolated and probed for *CLB2*. The same membrane was reprobed for *ACT1* as a loading control. (B) Clb2p-Cdc28p kinase activity was assessed by in vitro kinase assays by using histone H1 (HH1) as a substrate. Kinase assays were performed on immunoprecipitated Clb2p-HA Cdc28p complexes isolated from wild-type MT588 cells treated the same as in A. (C) Clb2-HA and Cdc28p levels in total cell lysate used for B were determined by Western blot analysis. (D) Stability of Clb2–HA/Cdc28p complexes was determined by immunoprecipitating Clb2-HAp from the samples used for B and C. Western blots were then probed with anti-Cdc28 and anti-HA antibodies. The band marked with * is IgG. W303 cells lacking the HA tag on Clb2p were used as a control. Similar results were obtained in at least three separate experiments.

lowing addition of 0.4 M NaCl, but with a substantial lag compared with repression of *CLB2* mRNA (Figures 2B and 5A). Thus, it appears that hypertonic stress affects *CLB2* transcription before its effects on Clb2p-Cdc28p kinase activity. Inhibition of Clb2p-Cdc28p activity was also observed when cells were stressed by 1 M sorbitol (our unpublished results).

To determine whether hypertonic stress had an effect on the level of Clb2 or Cdc28 protein, the amount of Clb2-HAp and Cdc28p present in total cell lysate was determined by Western blot analysis. The addition of 0.4 M NaCl did not affect the abundance of either protein (Figure 2C). Although the abundance of Clb2p and Cdc28p was unaffected by hypertonic stress, we were interested in determining whether the Clb2p/Cdc28p complex remained intact following treatment with 0.4 M NaCl. Anti-HA antibodies were used to immunoprecipitate Clb2-HAp. Western blots were probed with anti-HA and anti-Cdc28 antibodies. The amount of Cdc28p that coprecipitated with Clb2-HAp was not affected by hypertonic stress, indicating that the Clb2–HA/Cdc28p complex remained intact (Figure 2D).

Hypertonic Stress Causes a G2 Delay

The accumulation of 2N cells, the decrease in *CLB2* mRNA, and the inhibition of Clb2p-Cdc28p kinase activity all suggest that hypertonic stress causes a cell cycle delay in G2/M. To examine this possibility more directly, we studied the effect of osmotic stress on the timing of mitosis in synchronized cultures. Cultures enriched in G2 phase cells were obtained by first isolating small unbudded cells by centrifugal elutriation and then allowing the cells to grow synchronously until the population was >80% budded with only 10–20% of the cells having completed mitosis. The fraction of cells that had completed mitosis was scored as the total number of cells that had either two separated nuclei or had completed cell division (Lew and Reed, 1995). The culture was split in two and NaCl added to a final concentration of 0.4 M to half of the culture. Note that this regimen is in contrast to previous studies on the morphogenesis checkpoint, in which cells were perturbed well before bud emergence and completion of S phase (Lew and Reed, 1995; Sia *et al.*, 1996, 1998). Initial experiments indicated that NaCl-treated cells showed an ~30-min delay in the onset of mitosis compared with the control culture (our unpublished results).

To examine the effects of different regulatory protein mutations on the kinetics of cell cycle delay after osmotic stress, we used mutants that were defective in the osmoregulation response. The assumption behind this approach is that, like DNA damage-signaling pathways, osmotic stress-signaling pathways control two different responses, one delaying the cell cycle, and the other allowing adaptation to the stress. Thus, to determine the effects of different mutations on the kinetics of cell cycle delay per se, experiments are best done in a genetic background that prevents the adaptation to osmotic stress. Exposure of yeast to increases in the external osmolarity induces the synthesis and accumulation of glycerol, leading to a restoration of the osmotic gradient and resumption of cell growth (Brown, 1990; Ansell *et al.*, 1997). This glycerol-based osmoregulation is eliminated by deletion of *GPD1* and *GPD2,* enzymes responsible for catalyzing glycerol production, or deletion of HOG pathway genes that regulate expression of glycerol synthesis genes (Albertyn *et al.*, 1994a,b; Eriksson *et al.*, 1995). When a *gpd1*Δ *gpd2*Δ mutant was tested for cell cycle progression after osmotic stress, the mitotic index did not rise (Figure 3A), showing a sustained cell cycle arrest. It could be argued that the slow growth rate of these cells (Figure 3, A–E) in raffinose leads to reduced synchrony. However, the low number of cells that have completed mitosis (10–20%) coupled with the immediate rise in the mitotic index of no salt control samples to 100% within 120 min shows that these cultures are enriched for G2/M cells. Note also that S phase cells under osmotic stress appear to complete DNA synthesis before arresting at G2/M (Figure 1A), suggesting that any delay in cell cycle progression is therefore likely to be explained by a G2/M delay.

Swe1p Is Required for the Hypertonic Stress-induced Cell Cycle Delay

The results from the mitotic index experiments with *gpd1*Δ *gpd2*Δ cells suggest a cell cycle delay in G2/M in response to hypertonic shock. To determine what pathway(s) might sig-

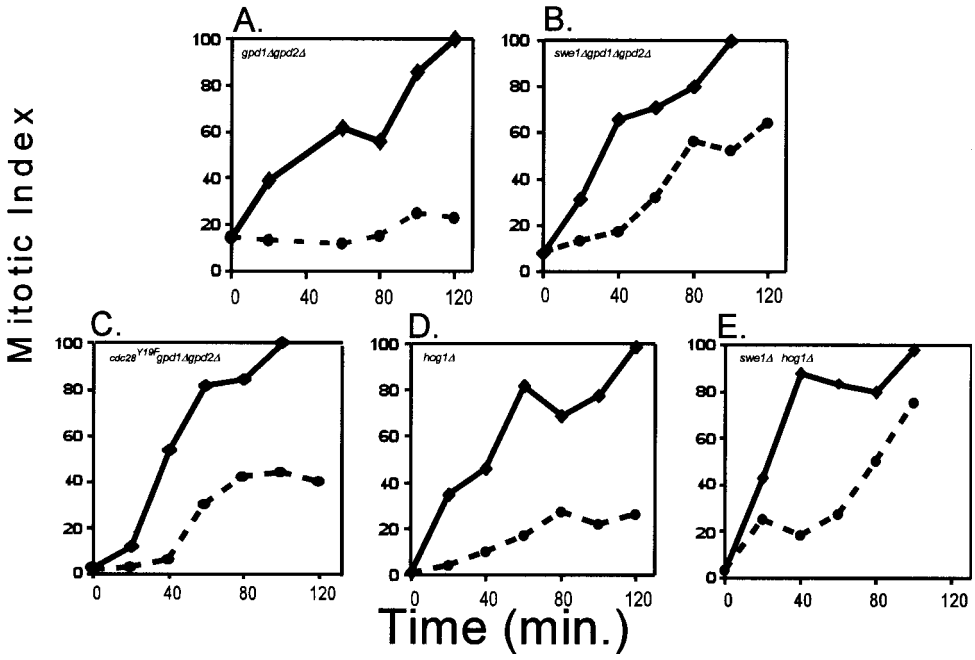


Figure 3. Hypertonic shock causes a G2 delay. For each experiment, cells were grown and eluted in YEP + raffinose to reduce the incidence of clumping and disruption of media flow. The synchronized culture was grown until ~80% budded. The culture was split and NaCl was added to one flask to a final concentration of 0.4 M (dashed line). The remaining flask served as a no-salt control (solid line). The mitotic index, an indication of the percentage of cells that have completed nuclear division, was determined as described (see MATERIALS AND METHODS). Time corresponds to minutes after addition of NaCl. (A) Cell cycle progression is halted when MT588 *gpd1*Δ *gpd2*Δ cells are stressed by 0.4 M NaCl. (B) *swe1*Δ *gpd1*Δ *gpd2*Δ cells do not halt cell cycle progression in response to osmotic stress, as indicated by the immediate increase in the mitotic index after the addition of 0.4 M NaCl. (C) Hypertonic stress causes a brief transient delay in *cdc28*^{Y19F}-HA *gpd1*Δ *gpd2*Δ cells. (D) Deletion of *HOG1* has a limited effect on the hypertonic stress-induced delay. (E) Response of *swe1*Δ *hog1*Δ cells to hypertonic stress was comparable to the response of *swe1*Δ *gpd1*Δ *gpd2*Δ cells. Similar results were obtained in at least three separate experiments.

nal the cell cycle delay, we examined the effects of hypertonic shock by using various mutant strains. Swe1p phosphorylates and inhibits Cdc28p (Booher *et al.*, 1993) and is required for a cell cycle delay in response to disruption of the normal organization of the actin cytoskeleton or the septin ring (Sia *et al.*, 1996; McMillan *et al.*, 1998; Barral *et al.*, 1999). Because hypertonic stress also disrupts the actin cytoskeleton (Chowdhury *et al.*, 1992; Brewster and Gustin, 1994) and apparently stabilizes Swe1p (Sia *et al.*, 1998), the role of Swe1p in the response to hypertonic shock was examined. To determine the effect of hypertonic stress on cell cycle progression in a cell lacking Swe1p, the timing of mitosis was determined in synchronous cultures of *swe1*Δ *gpd1*Δ *gpd2*Δ mutants following addition of 0.4 M NaCl. In the absence of Swe1p, the mitotic index increased immediately following hypertonic shock, suggesting a defective delay mechanism (Figure 3B). This result contrasts with the results from *gpd1*Δ *gpd2*Δ cells, where the mitotic index did not rise after the addition of NaCl (Figure 3A). Hypertonic stress did however, slow the rate of the increase in the mitotic index compared with the control.

The finding that deletion of *SWE1* caused a loss of the hypertonic stress-induced cell cycle delay suggests that Swe1p-mediated phosphorylation of Cdc28p is involved in this process. To further examine the role of Cdc28p phosphorylation, the timing of mitosis following addition of 0.4 M NaCl was determined using cells expressing a mutant Cdc28p that cannot be phosphorylated by Swe1p. In these *cdc28*^{Y19F}*-HA gpd1*Δ *gpd2*Δ cells, hypertonic stress caused a transient cell cycle delay, where the mitotic index did not rise for a period of ~30 min (Figure 3C). This response is different from that of *gpd1*Δ *gpd2*Δ and *swe1*Δ *gpd1*Δ *gpd2*Δ, suggesting that phosphorylation of Cdc28p may not be the only factor contributing to this stress response. Consistent with this hypothesis is the finding that Swe1p has a phosphorylation-independent role in triggering the morphogenesis checkpoint (McMillan *et al.*, 1999).

Because hypertonic stress causes the activation of the HOG pathway (Brewster *et al.*, 1993), we tested the role of the MAP kinase (MAPK) Hog1p in the hypertonic stress-induced G2/M delay. Hog1p is required for optimal recovery from hypertonic stress and activation of Hog1p induces glycerol accumulation through induction of *GPD1.* Thus, deletion of *HOG1*, like deletion of *GPD1* and *GPD2* inhibits the ability of cells to accumulate glycerol and restore the osmotic gradient (Brewster *et al.*, 1993). After addition of 0.4 M NaCl to *hog1*Δ mutants (Figure 3D), there was only a small increase in mitotic index over that seen in *gpd1*Δ *gpd2*Δ cells (Figure 3A). Analysis of cell cycle progression after hypertonic stress in *hog1*Δ *gpd1*Δ *gpd2*Δ triple mutants gave similar results to *hog1*Δ single mutants (our unpublished results). Thus, the addition of 0.4 M NaCl did not completely prevent an increase in the mitotic index in *hog1*Δ cells, but the effect of deleting *HOG1* was not nearly as strong as the effect of deleting *SWE1.* Deletion of *SWE1* in the *hog1*Δ background resulted in a mitotic index similar to *swe1*Δ mutants (Figure 3E). Thus, Swe1p appears to be the main effector of the mitotic delay.

Swe1-dependent Phosphorylation of Cdc28 and Hog1-dependent Inhibition of Cdc28

The results from Figure 3 suggest that Swe1p is an important component of a hypertonic stress-induced cell cycle delay.

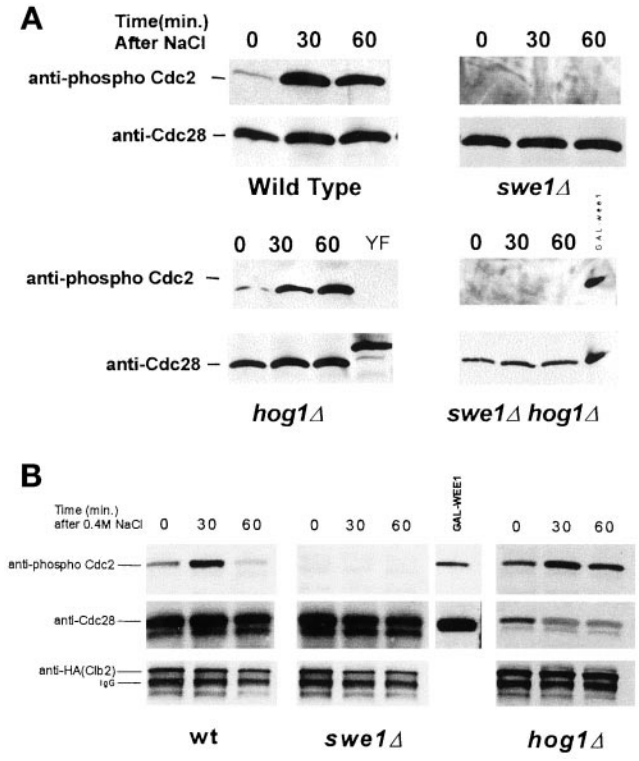


Figure 4. Hypertonic stress induces the phosphorylation of Cdc28p in a Swe1p-dependent manner. Asynchronous cultures of MT588 were stressed by the addition of 0.4 M NaCl. (A) Cdc28p was precipitated with $p13^{Suc1}$-agarose beads and analyzed by Western blot. Anti-phospho-Cdc2 was used to determine the phosphorylation state of Cdc28p (see MATERIALS AND METHODS). Membranes were stripped and reprobed with anti-Cdc28. A GAL: *wee1*$^+$ *mih1*Δ strain and a $Cdc28^{Y19F}$-HA strain were used as positive and negative controls, respectively. The decrease in mobility of the Y19F control is the result of the HA epitope tag. (B) Phosphorylation of Clb2-Hap-associated Cdc28p was examined by immunoprecipitating Clb2–HAp/Cdc28p complexes with anti-HA antibodies and protein A/G agarose beads. As a positive control, hyperphosphorylated Cdc28p from GAL: *wee1*$^+$ *mih1*Δ cells was coprecipitated with $p13^{Suc1}$-agarose beads. Western blots were probed with anti-phospho-Cdc2 antibodies and then stripped and reprobed with anti-Cdc28 and anti-HA antibodies. Results were consistent in three separate experiments.

As mentioned previously, Swe1p has been shown to phosphorylate Cdc28p (Booher *et al.*, 1993). To examine whether hypertonic shock induces Cdc28p phosphorylation by Swe1p, $p13^{Suc1}$-agarose beads were used to precipitate Cdc28p from cell lysates and the phosphorylation state of the coprecipitated Cdc28p determined by Western blot with anti-phospho-Cdc2 antibody. Hypertonic shock caused an increase in Cdc28p phosphorylation within 30 min, which was prevented by deletion of *SWE1* (Figure 4A). In contrast, deletion of *HOG1* did not block the hypertonic stress-induced tyrosine phosphorylation of Cdc28p. To examine more specifically the phosphorylation of Cdc28p in complex with Clb2p, Clb2p–HA/Cdc28p complexes were isolated by anti-HA immunoprecipitation. Immunoblots were probed with anti-phospho-Cdc2 antibodies and reprobed with anti-Cdc28 and anti-HA antibodies (Figure 4B). Consistent with the results from $p13^{Suc1}$ coprecipitation experiments, Clb2p associated Cdc28p was also phosphorylated in response to hypertonic shock. In *hog1*Δ mutants, the degree of Cdc28p phosphorylation in Clb2p–HA/Cdc28p complexes after hypertonic stress was similar to that of wild type.

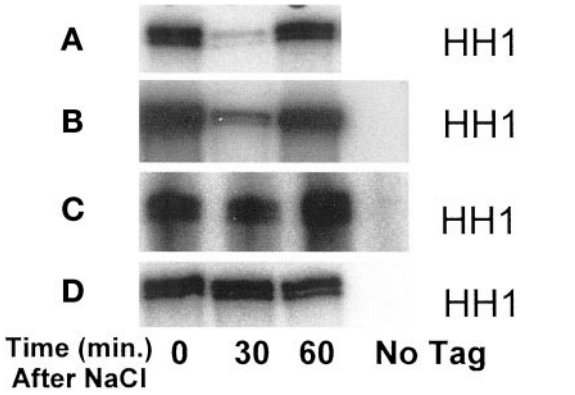


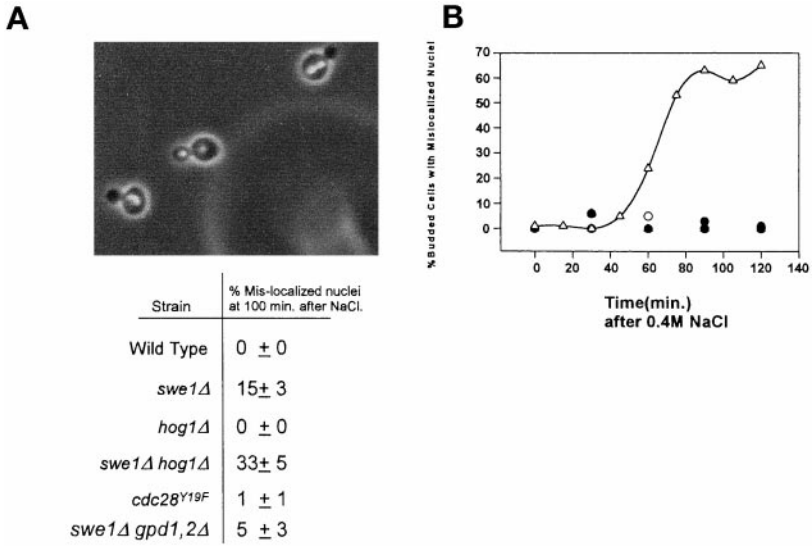
Figure 5. Inhibition of Clb2p-Cdc28p kinase activity by exposure to 0.4 M NaCl. Wild-type MT588 (A), *swe1*Δ (B), *hog1*Δ (C), and *hog1*Δ *swe1*Δ (D) strains were grown to log phase and stressed by the addition of NaCl to 0.4 M. Clb2p–Cdc28p complexes were immunoprecipitated and assayed for activity before and after the addition of NaCl. A strain lacking the HA tag on Clb2p was used as a control. Similar results were observed in at least three independent experiments.

The hypertonic shock-induced, Swe1p-dependent phosphorylation of Cdc28p, and the Swe1p-dependent cell cycle delay suggested that Swe1p might also be required for the observed inhibition of Clb2p-Cdc28p kinase activity after hypertonic stress (Figure 2B). To test this idea, the activity of Clb2p–Cdc28p complexes from *swe1*Δ cultures was determined after addition of 0.4 M NaCl. Surprisingly, the activity of Clb2p–Cdc28p complexes isolated from *swe1*Δ cells was inhibited by 0.4 M NaCl to an extent similar to that observed for wild type (Figure 5B).

We next determined whether the inhibition of Clb2p-HA-associated Cdc28p kinase activity under hypertonic stress would be affected in a *HOG1* mutant. Deletion of *HOG1* blocked the inhibition of kinase activity following hypertonic shock (Figure 5C). Swe1p did not significantly contribute to inhibition of Clb2p-Cdc28p because there was no additional elevation of Clb2-Cdc28 kinase activity in *hog1*Δ *swe1*Δ double mutants (Figure 5D). Taken together, the above results suggest that following hypertonic shock, the inhibition of Clb2p-Cdc28p activity does not strongly correlate with mitotic delay, and that the inhibition of Clb2-Cdc28p does not solely depend upon tyrosine phosphorylation.

Swe1 and Hog1 Prevent Mislocalization of Mitosis under Hypertonic Stress

In *S. cerevisiae*, mitosis takes place at the bud neck, resulting in the segregation of a single nucleus to the mother cell, and a single nucleus to the daughter cell. Wild-type cells exposed to hypertonic stress show no defects in segregation of nuclei



Strain	% Mis-localized nuclei at 100 min. after NaCl.
Wild Type	0 ± 0
swe1Δ	15± 3
hog1Δ	0 ± 0
swe1Δ hog1Δ	33± 5
cdc28^{Y19F}	1 ± 1
swe1Δ gpd1,2Δ	5 ± 3

Figure 6. (A) Mislocalization of nuclei in cells that are unable to delay in G2. Cells were treated as described in Figure 3. 4,6-Diamidino-2-phenylindole was used to stain the nuclei (*swe1*Δ *hog1*Δ cells shown). The same samples were also used to determine mitotic indices (Figure 3). The percentage of cells with separated nuclei that had not properly segregated (both nuclei in the mother cell) was determined ±SD from a minimum of three independent experiments. (B) Nuclear segregation is abnormal in *hog1*Δ cells following salt stress after release from alpha mating factor. Wild-type and *hog1*Δ cultures were arrested in G1 with alpha factor and released into fresh media. After 100 min, the culture was stressed by the addition of 0.4 M NaCl. The appearance of mislocalized nuclei in budded cells following the addition of NaCl was plotted versus time. A representative experiment with wild-type cells with or without salt (○ and ●, respectively) and *hog1*Δ cells with and without salt (△ and ▲, respectively) is shown here.

to mother and daughter cells. However, as shown previously (Lew and Reed, 1995), hypertonic stress under conditions where the Swe1p checkpoint is overridden in wild-type cells, by the overexpression of Clb2p, results in the accumulation of binucleated mother cells under conditions of hypertonic stress. We found that deletion of *SWE1* also caused cultures to accumulate binuclear mother cells under conditions of hypertonic stress (Figure 6A). Consistent with our finding that elimination of Cdc28p tyrosine phosphorylation does not completely abrogate the mitotic delay in response to hypertonic stress, we found that nuclear mislocalization occurred only rarely in *cdc28*^{Y19F} cells under hypertonic stress conditions (Figure 6A). Although deletion of *HOG1* has little effect on proper nuclear segregation in elutriation synchronized salt stressed cells, a *hog1*Δ *swe1*Δ double mutant accumulates more cells with mislocalized nuclei than a *swe1*Δ culture (Figure 6A). A role for Hog1p can be more easily seen when *hog1*Δ cells are exposed to hypertonic stress following release from a mating pheromone-induced G1 arrest. In these cultures ~70% of large budded cells had two nuclei localized to the mother cell (Figure 6B). The increase in mislocalized nuclei in a *swe1*Δ *hog1*Δ double mutant could be the result of the inability to restore the osmotic gradient caused by a disrupted HOG pathway. However, a *swe1*Δ *gpd1*Δ *gpd2*Δ culture treated in the same manner did not give rise to the same increase in mislocalized nuclei (Figure 6A). Thus, Hog1p appears to share overlapping functions with Swe1p in enforcing proper nuclear segregation under conditions of hypertonic stress.

DISCUSSION

Our study shows that hypertonic stress induces a cell cycle delay in both G1 phase and G2 phase. The G2 delay is correlated with both a Swe1p-dependent increase in tyrosine phosphorylation of Cdc28p and a Hog1p-dependent decrease in kinase activity of the Clb2p–Cdc28p complex. Comparison of the phenotypes of *swe1*Δ strains to that of *hog1*Δ strains suggests that the tyrosine phosphorylation state of Cdc28p rather than its actual kinase activity appears to be more important in regulating cell cycle progression. However, Hog1p may also have effects on cell cycle progression as revealed by the increase in nuclear mis-segregation in *hog1*Δ *swe1*Δ double mutants.

Comparison of the Hypertonic Stress Response and Activation of the Morphogenesis Checkpoint

Mutational or chemical disruption of the actin cytoskeleton in cells with small buds triggers a G2 arrest termed the morphogenesis checkpoint (Lew and Reed, 1995; Sia *et al.*, 1996; McMillan *et al.*, 1998; Barral *et al.*, 1999). In this pathway, a family of Swe1p inhibitory kinases monitors proper septin ring assembly, and in the absence of a septin ring is held in an inactive state, thereby allowing Swe1p to phosphorylate and inhibit Clb–Cdc28 complexes (Barral *et al.*, 1999). Once septin assembly occurs, bud morphogenesis is initiated, and Swe1 is inactivated. Deletion of *SWE1* abrogates the checkpoint response (Lew and Reed, 1995). Hypertonic stress also disrupts the actin cytoskeleton (Chowdhury *et al.*, 1992), and consequently activates the morphogenesis checkpoint (Lew and Reed, 1995; Sia *et al.*, 1998).

Through the use of synchronized cultures, we have found that Swe1p may also impose a hypertonic stress-induced cell cycle delay. This mechanism appears to be sensitive to stresses that occur later in the cell cycle than those that can trigger the morphogenesis checkpoint (McMillan *et al.*, 1998). *swe1*Δ cells exposed to hypertonic stress appear to enter mitosis with little or no initial delay (Figure 3), although the loss of Swe1p does not fully restore cell cycle kinetics, suggesting that other factors may also contribute to the response. The effects of hypertonic shock on cell cycle progression are similar to those caused by activation of the morphogenesis checkpoint: Clb2p-Cdc28p kinase activity is inhibited (Lew and Reed, 1995), the accumulation of *CLB2* mRNA is delayed (Sia *et al.*, 1996), and tyrosine phosphorylation of Cdc28p is increased (Lew and Reed, 1995). However, the osmotic stress-induced G2 delay and the morpho-

genesis checkpoint are not identical. In the latter case, a delayed induction of *CLB2* mRNA and protein correlates with a delay in entry into mitosis. In contrast, increased osmolarity induces a large drop in *CLB2* mRNA, but has little effect on Clb2p levels (Figure 2). This difference is likely to derive in part from the timing of the actin cytoskeleton-disrupting stress relative to the state of a Clb1/2p-Cdc28p positive feedback loop that regulates entry of cells into mitosis (Amon *et al.*, 1993). Activation of the morphogenesis checkpoint early in the cell cycle prevents the onset of a *CLB2* positive feedback loop. In these experiments, however, the hypertonic stress is occurring later in the cell cycle after the feedback loop has already been established. Because Clb2p is stable in post-G1 phase cells, Clb2p persists even though *CLB2* expression and Clb2p-Cdc28p kinase activity are diminished by hypertonic stress (Amon *et al.*, 1993). In addition, hypertonic stress induces tyrosine phosphorylation of Cdc28p within 30 min (Figure 4), whereas a significant increase in Cdc28p phosphorylation in a *cdc24-1* strain is not detected until 2–3 h after a shift to the nonpermissive temperature (Lew and Reed, 1995).

The morphogenesis checkpoint monitors the septin ring/actin cytoskeleton only in G1 cells and early S phase cells with a very small bud, but not later in the cell cycle (Lew and Reed, 1995; McMillan *et al.*, 1998). In part, this is because the abundance of Swe1p is controlled by cell cycle-regulated transcription and ubiquitin-dependent proteolysis, which results in higher expression in G1 and early S phase cells (McMillan *et al.*, 1998; Sia *et al.*, 1998). Although this finding appears at odds with the Swe1p dependence of the hypertonic delay in G2 phase cells, residual Swe1 protein persists in cells that are refractory to perturbation of the actin cytoskeleton (McMillan *et al.* 1998). The lower level of Swe1p in G2 phase may be sufficient to mediate the hypertonic stress-induced delay but not the morphogenesis checkpoint delay. Consistent with this hypothesis is the recent finding that Swe1p is present and important at later phases (G2/M) of the cell cycle (Sreenivasan and Kellogg, 1999). Thus, although the morphogenesis checkpoint and the cell cycle delay caused by hypertonic shock share Swe1p as a common component, the signals that regulate Swe1 may be different in each response. The dependence of the hypertonic stress response on components upstream of Swe1 remains to be determined.

Finally, we note that hypertonic stress delays the initiation of DNA synthesis in G1 phase cells, as shown by the depletion of S phase cells in asynchronous cultures and by the delayed onset of DNA replication in cultures released from mating pheromone arrest. In contrast, perturbation of septin/actin function does not affect the onset of DNA replication (Lew and Reed, 1995). Taken together, the above-mentioned results suggest that hypertonic stress does disrupt the actin cytoskeleton and, like activation of the morphogenesis checkpoint, does trigger a Swe1p-dependent cell cycle delay in response to osmotic stress early in the cell cycle. However, unlike the morphogenesis checkpoint, hypertonic shock also delays cell cycle progression at later points in the cell cycle.

Role of the HOG Pathway

Our data show that Hog1p is required for the decrease in Clb2p-Cdc28p kinase activity following hypertonic stress. Furthermore, deletion of *HOG1* increases the fraction of *swe1*Δ cells that accumulate two nuclei in the mother cell after hypertonic stress. Taken together, these data suggest that Hog1p might work together with Swe1p to impose a cell cycle delay in G2 phase, which prevents aberrant nuclear segregation. However, our data are also consistent with Hog1p playing a role in the proper orientation of the mitotic spindle. In *hog1*Δ mutants, Swe1p might therefore be important to halt cell cycle progression until the spindle is properly aligned. This would account for the increase in mislocalized nuclei in elutriation synchronized *hog1*Δ *swe1*Δ double mutants exposed to a hypertonic stress (Figure 6A). Surprisingly, when *hog1*Δ cells are exposed to hypertonic stress following release from mating pheromone, ~70% of large-budded cells had two nuclei localized within the mother cell even though Swe1p should be present in these cells (Figure 6B). However, exposure to mating pheromone might be affecting Swe1p levels or activity in these experiments.

The mechanisms whereby Swe1p and Hog1p affect cell cycle progression are unclear and somewhat contrary to expectations. First, the Hog1p-dependent inhibition of Clb2p-Cdc28p kinase activity (Figure 5) is puzzling in light of the finding that cell cycle progression is significantly slowed, if not completely delayed, even though Clb2p-Cdc28p activity remains high following hypertonic stress. In cells with an intact Swe1p pathway, Hog1p-dependent effects may simply be too subtle to detect by the mitotic progression assay (see above). Conversely, although Swe1p does stimulate tyrosine phosphorylation of Cdc28p, and is necessary for the hypertonic stress-induced delay, these events do not correlate with inhibition of Clb2p-Cdc28p activity. Thus, tyrosine phosphorylation of Cdc28p, and not inhibition of Clb2p-Cdc28p activity, appears to be a more critical factor for the cell cycle delay. However, there does appear to be phosphorylation-independent effects of Swe1p as seen by the partial cell cycle delay observed in a $CDC28^{Y19F}$ strain.

Previous work has shown that Clb2p-Cdc28p kinase activity is also depressed in cells arrested by the morphogenesis checkpoint, but in this instance decreased amount of Clb2 protein and not phosphorylation of Cdc28p seems to account for most of the reduction in kinase activity (Lew and Reed, 1995). Because it appears that tyrosine phosphorylation does not dramatically alter Cdc28p activity, it may be that Swe1-dependent phosphorylation alters the localization and/or assembly of Cdc28p complexes with other factors under conditions of hypertonic stress. The mechanism whereby the Hog1p pathway contributes to Cdc28p inhibition is also not understood at this time.

Regulation of the Cell Cycle by MAPK Pathways

Numerous examples suggest that MAPK signaling is a common means to regulate cell cycle progression. In budding yeast, the MAPK Fus3p activates the CDK inhibitor Far1p, which eliminates Cln-Cdc28p activity and causes G1 arrest in preparation for mating (Peter *et al.*, 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994). In fission yeast, the stress-activated MAPK pathway based on the Hog1p homolog *Sty*I (also called Spc1 or Phh1) positively regulates cell cycle progression by an unknown mechanism (Shiozaki and Russell, 1995). In animal cells, the embryonic cell cycle is controlled by MAPK pathways that mediate hormone-

dependent stimulation of the cell cycle. Finally, the Ras-Erk MAPK pathway helps couple growth factor stimulation to G1 progression in mammalian tissue cultures cells (Weber *et al*., 1997). The mechanisms that couple MAPK activity to the cell cycle machinery, as in the Hog1p-mediated inhibition of Cdc28p activity, represent an important means by which cell division is controlled by environmental cues.

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