

GSK-3 kinase Mck1 and calcineurin coordinately mediate Hsl1 down-regulation by Ca²⁺ in budding yeast

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The Ca²⁺-activated pathways of *Saccharomyces cerevisiae* induce a delay in the onset of mitosis through the activation of Swe1, a negative regulatory kinase that inhibits the Cdc28–Clb complex. Calcineurin and Mpk1 activate Swe1 at the transcriptional and post-translational level, respectively, and both pathways are essential for the cell cycle delay. Our genetic screening identified the *MCK1* gene, which encodes a glycogen synthetase kinase-3 family protein kinase, as a component of the Ca²⁺ signaling pathway. Genetic analyses indicated that Mck1 functions downstream of the Mpk1 pathway and down-regulates Hsl1, an inhibitory kinase of Swe1. In medium with a high concentration of Ca²⁺, Hsl1 was delocalized from the bud neck and destabilized in a manner dependent on both calcineurin and Mck1. Calcineurin was required for the dephosphorylation of autophosphorylated Hsl1. The E3 ubiquitin ligase complex SCF^{Cdc4}, but not the anaphase-promoting complex (APC), was essential for Hsl1 destabilization. The Ca²⁺-activated pathway may play a role in the rapid inactivation of Hsl1 at the cell cycle stage(s) when APC activity is low.

Keywords: calcineurin/G₂ delay/GSK-3 family kinase/ MAP kinase/SCF complex

Introduction

In eukaryotic cells, a change in the cytosolic Ca²⁺ concentration plays a key regulatory role in diverse cellular processes, such as T-cell activation, muscle contraction and neurotransmitter release (Clapham *et al.*, 1995). In the yeast *Saccharomyces cerevisiae*, Ca²⁺ has been implicated in stress-induced expression of ion transporter genes (Haro *et al.*, 1991; Cunningham and Fink, 1994, 1996), bud formation (Mazzoni *et al.*, 1993; Zarzov *et al.*, 1996; Gray *et al.*, 1997) and viability upon pheromone-induced growth arrest (Cyert and Thorner, 1992). More recently, Ca²⁺ was also implicated in the regulation of cell cycle progression in G₂ phase (Mizunuma *et al.*, 1998). In *S. cerevisiae*, Swe1 kinase specifically inhibits a G₂ form of Cdc28 by phosphorylating it at Tyr19 (Booher *et al.*, 1993), and delays the onset of mitosis. The cell cycle regulation by Ca²⁺ is accom-

plished through the activation of calcineurin and the Mpk1 MAP kinase cascade, and these two pathways activate Swe1. Although the physiological role of Ca²⁺-mediated cell cycle regulation is not yet well understood, this regulation has been implicated in a mechanism that may operate under conditions where cells encounter a stress that may cause membrane stretching (Mizunuma *et al.*, 1998). The effect of Ca²⁺ on cell cycle regulation is particularly obvious in a $\Delta zds1$ genetic background, in which the *SWE1* expression level is higher than in wild-type cells. In this signaling process, calcineurin and the Mpk1 cascade up-regulate Swe1 at the transcriptional and post-translational level, respectively, and both of these pathways are required to mediate the cell cycle delay. The Mpk1 pathway activates Swe1 by inhibiting Hsl1, a Nim1-related kinase that negatively regulates Swe1 and leads to a delay in the entry into mitosis (Ma *et al.*, 1996; Tanaka and Nojima, 1996; Mizunuma *et al.*, 1998). However, the molecular mechanism underlying Hsl1 regulation by the Mpk1 pathway is as yet unknown.

Selective protein degradation is a commonly employed mechanism for the control of protein abundance. Ubiquitin-dependent proteolysis has emerged as a key mechanism for the regulation of many cellular processes, including cell cycle transitions (for reviews see Hochstrasser, 1996; Patton and Willems, 1998). The ubiquitin degradation pathway is a multistep process in which ubiquitin is covalently attached to a protein substrate by a series of enzymatic reactions involving a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitin ligases confer substrate specificity to the ubiquitylation reaction. One class of ubiquitin ligase that controls cell cycle progression in yeast and higher eukaryotes is a multi-subunit complex known as the anaphase-promoting complex (APC). APC activity itself is regulated, being active from anaphase onset through G₁, but then inactive in S, G₂ and early M phase (Amon *et al.*, 1994; Brandeis and Hunt, 1996; Amon, 1997; Jaspersen *et al.*, 1998). Another major ubiquitin ligase is the SCF. The F-box domain proteins act as the specificity-determining subunit of the SCF complex (Patton *et al.*, 1998). A central feature of the SCF-dependent ubiquitylation is its selectivity for phosphorylated substrates. Cdc6, Gcn4, Far1 and Sic1 are ubiquitylated and degraded in a manner dependent on a kinase that phosphorylates the substrate. The F-box protein Cdc4 interacts with phosphorylated substrate and recruits it to the ubiquitin ligase complex (SCF^{Cdc4}) composed of Cdc4, Skp1 and Cdc53 (Feldmann *et al.*, 1997; Skowrya *et al.*, 1997). Although ubiquitin-dependent proteolysis is involved in cell cycle control, the relationship of this system to the Ca²⁺-activated pathways is not known.

To identify a novel component(s) of the Ca²⁺ signaling pathways linked to cell cycle regulation, mutant strains that disrupt the signaling pathways were isolated and characterized. In this screening, *MCK1*, encoding a glycogen synthetase kinase-3 (GSK-3) family protein kinase, was identified as a component of this regulatory pathway. We demonstrate that Mck1 is a downstream regulator of the Mpk1 pathway. We propose a model in which Mck1 and calcineurin cooperatively regulate the delocalization of Hsl1 from the bud neck and subsequent SCF^{Cdc4}-mediated destabilization to trigger the Swe1 activation.

Results

Identification of mutations that disrupt Ca²⁺-induced G₂ arrest and polarized bud growth

In previous work, we showed that *ZDS1* null mutant ($\Delta zds1$) cells grown in medium containing a high concentration of CaCl₂ (>50 mM) were delayed in the G₂ phase and displayed polarized bud growth, due to the activation of cellular Ca²⁺ signaling pathways (Mizunuma *et al.*, 1998). This delay is mediated by the activation of Swe1, a negative regulatory kinase of the Cdc28–Clb complex. Two pathways, calcineurin and the Mpk1 MAP kinase cascade, are implicated in this event, and both pathways are indispensable for Ca²⁺-triggered growth arrest. These findings indicated that a defect in either branch of the Ca²⁺ signaling pathways would overcome the growth arrest. To investigate the detailed mechanism of cell cycle arrest, we performed a screen for mutants that displayed a defect in this process. The $\Delta zds1$ cells are unable to grow, and show hyperpolarized bud growth on solid medium containing 200–300 mM CaCl₂, whereas mutants with a defect in these pathways (e.g. $\Delta cnb1$ or $\Delta mpk1$) can grow well and show normal morphology. Our screening procedures comprised two steps. In the first screening, colonies of suppressor mutants with increased tolerance to Ca²⁺ were selected, which arose spontaneously from the $\Delta zds1$ strain on YPD plates containing 200 or 300 mM CaCl₂. In the second screening, the mutants restored to normal morphology on Ca²⁺ medium were selected by microscopic observation, because the mutants with the aberrant morphology may have gained an increased tolerance to Ca²⁺ by mutations in other mechanisms. By genetic crosses, the recessive Ca²⁺-insensitive mutants were classified into 14 complementation groups designated *scz1–scz14* (for suppressor of Ca²⁺-induced abnormalities of *zds1*). Details of the characterization of the mutants will be described elsewhere. In this report, we focused on characterizing the temperature-sensitive *scz10* mutant.

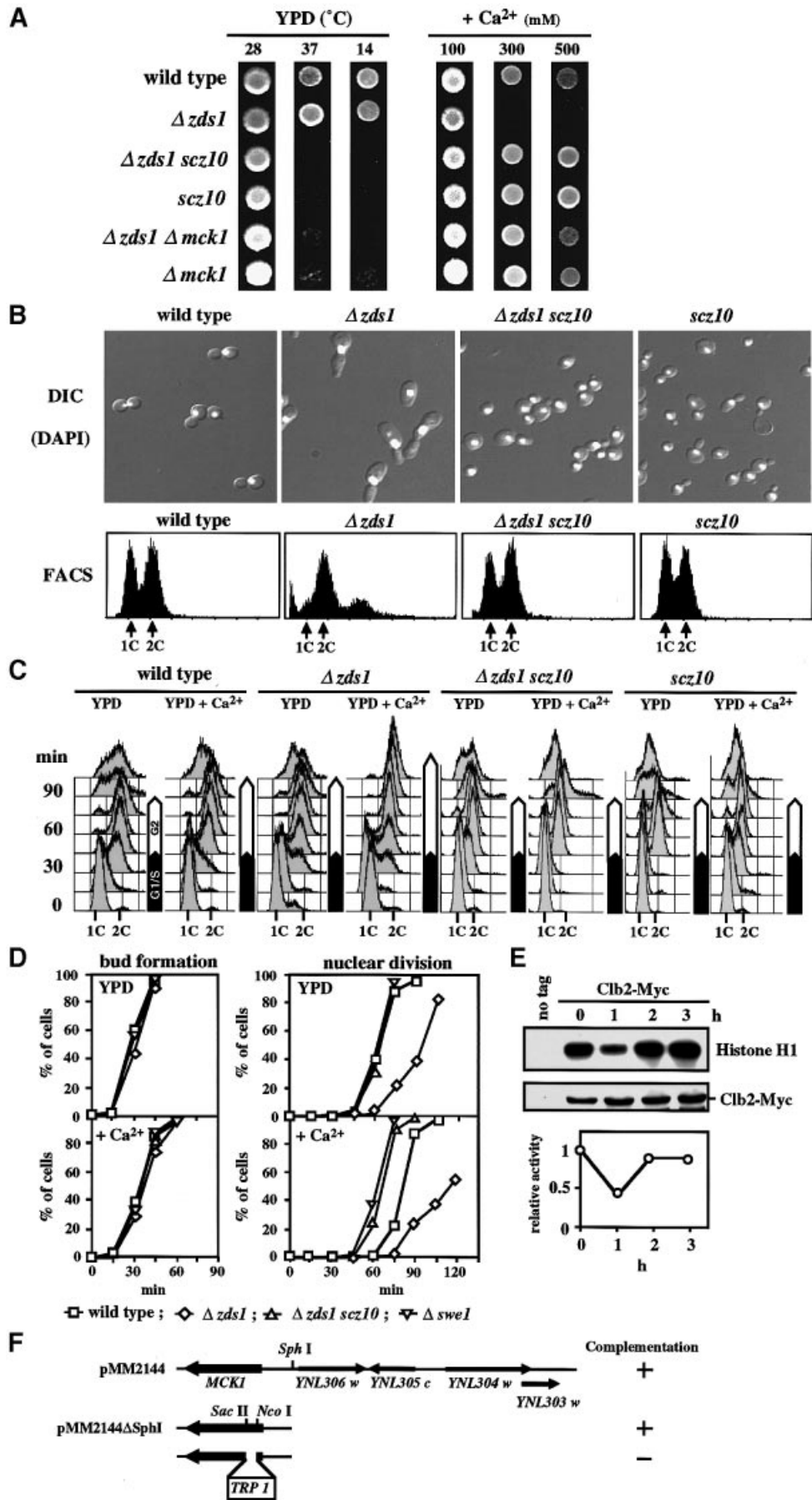
On solid medium containing 300 mM CaCl₂, the $\Delta zds1$ strain failed to grow, exhibiting an aberrant morphology, and were suppressed by the additional mutation *scz10* (Figure 1A and B). To confirm that the *scz10* mutation can overcome the Ca²⁺-induced cell cycle delay, we examined cell cycle progression by using the G₁-synchronous culture treated with α -factor. The synchronized cells of wild type, $\Delta zds1$, $\Delta zds1$ *scz10* and *scz10* were released into YPD medium with or without 50 mM CaCl₂. After the release, the DNA content, bud formation and nuclear division in synchronized cell cultures were determined (Figure 1C and D). Bud formation was slightly delayed by Ca²⁺, but

there was no significant difference among the strains (Figure 1D). No obvious delay in S phase by Ca²⁺ was observed in these strains (Figure 1C). However, compared with wild type, the $\Delta zds1$ cells were delayed in the onset of mitosis by 15 min in YPD medium (without added Ca²⁺) and this delay was prolonged to 30–45 min by Ca²⁺. It was noteworthy that a significant Ca²⁺-induced delay (15 min) in the onset of mitosis was observed with wild-type cells using this procedure. In contrast, Ca²⁺ caused no significant delay in the onset of mitosis in $\Delta zds1$ $\Delta scz10$ cells (Figure 1C and D). These data indicated that the *scz10* mutation suppressed the G₂ delay of $\Delta zds1$ cells. They also indicated that Ca²⁺ can induce the G₂ delay even in wild-type cells. In fact, we found by Clb2–Cdc28-dependent H1 kinase assay that the Cdc28 kinase activity was inhibited in an asynchronous cell culture of wild-type cells after 1 h of shift to high Ca²⁺ conditions (Figure 1E). The inhibition was transient and was restored with prolonged incubation. In addition to these suppressor phenotypes, the *scz10* single mutant exhibited temperature sensitivities at >37°C and <14°C (Figure 1A).

To identify the *scz10* gene, a centromeric genomic library was introduced into the *scz10* strain and one plasmid that suppressed the temperature sensitivity at 37°C was recovered. The genomic DNA insert in the cloned plasmid pMM2144 was subjected further to restriction mapping and complementation analysis (Figure 1F). Partial DNA sequencing of the complementing region revealed that it contained the *MCK1* gene (YNL307c ORF) encoding a protein kinase belonging to the GSK-3 family (Neigeborn and Mitchell, 1991; Shero and Hieter, 1991). Linkage of *MCK1* to the *SCZ10* locus was confirmed by tetrad analysis. In fact, *MCK1* disruption ($\Delta mck1$) suppressed the phenotypes of $\Delta zds1$ cells in the medium containing CaCl₂ (Figure 1A). The low and high temperature sensitivities of the *scz10* and $\Delta mck1$ mutants were similar to those previously reported for $\Delta mck1$ mutants (Shero and Hieter, 1991; Puziss *et al.*, 1994). Since this gene was described previously, we shall hereafter refer to *SCZ10* as *MCK1* and to the *scz10* mutant allele as *mck1-1*. The *mck1-1* allele, in comparison with the $\Delta mck1$ allele, exhibited qualitatively similar, but significantly more potent suppressor effects on the Ca²⁺-sensitive phenotypes of $\Delta zds1$, and increased sensitivities to extreme temperatures (Figure 1A). We further identified the mutation site of the *mck1-1* gene amplified by PCR. A single base deletion (G) at position +706 of the coding region was found. This deletion caused a frameshift and led to the appearance of a stop codon at position +747 of the 1128 bp open reading frame. The data showing that a catalytically inactive, truncated protein is expressed from the *mck1-1* gene are given below (Figure 5C). The nature of the truncated Mck1 may explain the stronger phenotypes of the *mck1-1* mutation than of a $\Delta mck1$ deletion allele (see Discussion). For this reason, we used the *mck1-1* mutant allele in most of the subsequent experiments.

Mck1 is a downstream component of the Mpk1 branch of the Ca²⁺ signaling pathways and down-regulates Hsl1 kinase

We next examined whether Mck1 functions in the calcineurin branch or the Mpk1 branch of the Ca²⁺ signaling pathways. The activation of one of these



branches by overexpression of *Mpk1* or *Cmp2ΔC*, a hyperactive form of the calcineurin catalytic subunit (Garrett-Engele *et al.*, 1995), on a *Δzds1* background leads to polarized bud growth and G₂ delay (Mizunuma *et al.*, 1998). If a mutation is located downstream of the activated pathway, the physiological effects will not be induced by the overexpression due to the interruption of this signal. On the basis of this assumption, the plasmid containing *MPK1* (pGAL::MPK1) or *CMP2ΔC* (pGAL::CMP2ΔC) placed under the control of the *GAL* promoter was introduced into the *Δzds1 mck1-1* strain. The overexpression of *CMP2ΔC*, but not *MPK1*, induced physiological changes, suggesting *Mck1* to be downstream of the *Mpk1* pathway (Figure 2A).

To examine whether *Mck1* induces the G₂ delay, we examined the effects of *MCK1* overexpression on the *Δzds1* background. Similarly to *MPK1*, *MCK1* overexpression led to a severe G₂ delay (Figure 2B). Previous genetic data indicated that the *Mpk1* cascade regulates the *Swe1* kinase through down-regulation of the *Hsl1* kinase, a negative regulator of *Swe1* (Mizunuma *et al.*, 1998). We therefore examined whether *Mck1* functions through the regulation of *Hsl1*. If *Mck1* activates *Swe1* through *Hsl1* regulation, *Mck1* overexpression should not enhance the G₂ delay in *Δhsl1* cells as found for *MPK1*. This was indeed the case (Figure 2B). The finding that the *mck1-1* mutation could not rescue the Ca²⁺-induced G₂ delay in *Δhsl1* cells supported the above genetic relationship (data not shown). Furthermore, overexpression of *MCK1* on a *Δzds1 Δmpk1* (Figure 2B), but not on a *Δswe1* background (data not shown) caused a strong G₂ delay, confirming that *MCK1* functions downstream of *MPK1*. Consistent with the notion that both *MPK1* and *MCK1* function in a common pathway, the suppressive effect of a *Δmpk1 mck1-1* double mutation on the phenotype of *Δzds1* was no more severe than those of the corresponding single mutations (data not shown). These genetic data established a cascade series of kinases, in which *Mpk1* activates *Mck1*, which in turn down-regulates *Hsl1* to induce *Swe1* activation (Figure 2C).

Consistent with the genetic results, northern blot analysis demonstrated that *Mpk1* is important for the induction of *MCK1* mRNA by Ca²⁺, indicating that *Mpk1* positively regulates *MCK1* at the transcriptional level (Figure 3).

The regulation of *Hsl1* by *Mck1* is not at the transcriptional level

To address whether *Mck1* down-regulates *HSL1* at the transcriptional level, we determined the level of *HSL1* mRNA by northern blot analysis in α -factor-synchronous cell cultures in the presence or absence of 50 mM CaCl₂ added at time 0 (Figure 4A). Quantification of cell morphology confirmed that >80% of the treated cells were arrested in G₁. For comparison, the level of *SWE1* mRNA was determined. The level of *SWE1* mRNA fluctuates during the cell cycle, peaking at G₁/S and declining in G₂/M (Ma *et al.*, 1996; Mizunuma *et al.*, 1998). In medium containing CaCl₂, a high level of *SWE1* mRNA was sustained in G₂/M by calcineurin (Mizunuma *et al.*, 1998). As previously reported, the level of *HSL1* mRNA fluctuated during the cell cycle in a manner similar to that of *SWE1* mRNA (Tanaka and Nojima, 1996). However, in contrast to *SWE1* mRNA, the oscillation of *HSL1* mRNA in the wild-type strain was not significantly altered by Ca²⁺. Moreover, the fluctuation pattern was unaffected by the presence or absence of the *MCK1* gene. These results indicated that *HSL1* is not regulated by Ca²⁺ at the transcriptional level, and the regulation of *Hsl1* by *Mck1* is at the post-transcriptional level.

Mck1 and calcineurin regulate *Hsl1* abundance

We next examined by immunoblot analysis whether Ca²⁺ down-regulates *Hsl1* abundance. For this purpose, we constructed strains containing a chromosomally integrated gene coding for three copies of the influenza virus hemagglutinin (HA) epitope-tagged *Hsl1*. The HA-tagged *Hsl1* suppressed the Ca²⁺-sensitive phenotype of the *Δhsl1* strain, suggesting that the construct is fully functional *in vivo*. We compared the levels of *Hsl1* in an asynchronous cell culture in medium with 100 mM CaCl₂. Cell extracts were prepared, separated by SDS-PAGE and detected by immunoblotting using antibodies directed against the HA epitope (Figure 5A). As previously reported, *Hsl1* protein migrated as a broad band, in which the slower migrating species were thought to correspond to the autophosphorylated, active isoform (Barral *et al.*, 1999). Because of this property, the *Hsl1* phosphorylation status provides a useful indicator of kinase activity (Barral *et al.*, 1999). Remarkably, the HA-*Hsl1* levels of wild-type and *Δzds1* cells decreased upon a shift to medium with CaCl₂, and the HA-*Hsl1* level dropped to about <10% of the original 1 h after the shift (Figure 5A). The rate of the Ca²⁺-induced decrease in

Fig. 1. The *scz10* mutation suppresses various phenotypes of the *Δzds1* strain. (A) Effect of *scz10* (*mck1-1*) on the growth of the *Δzds1* mutant strain on solid medium. Wild-type (DHT22-1b), *Δzds1* (YAT1), *Δzds1 scz10* (YMM2144), *Δzds1Δmck1* (YMM69), *scz10* (YMM22) or *Δmck1* (YMM68) cells were spotted on YPD plates and grown at 28°C (2 days), 37°C (2 days) or 14°C (4 days), or on YPD plates supplemented with the indicated concentration of CaCl₂ and incubated at 28°C for 2 days. (B) Cell morphology (DIC), DAPI fluorescence image and flow cytometry analysis of PI-stained cells (FACS: 1C, one DNA copy; or 2C, two DNA copies) of various strains after 6 h of incubation with 100 mM CaCl₂ at 28°C. (C) DNA content during cell cycle progression in various strains. Early log phase growing cells (OD₆₀₀ of 0.2–0.3) of DHT22-1b, YAT1, YMM2144 or YMM22 strains were synchronized with α -factor in G₁, and resuspended in YPD or YPD plus 50 mM CaCl₂. Samples were taken 15 min after removing α -factor. (D) Quantification of the cumulative percentage of bud formation and nuclear division in the cell cultures prepared as in (C). For comparison, the data with *Δswe1* (YMM5-8c) are shown. Open squares, DHT22-1b; open diamonds, YAT1; open triangles, YMM2144; inverted open triangles, YMM5-8c. (E) Inhibition of *Clb2*–*Cdc28* activity by CaCl₂ *in vivo*. CaCl₂ was added to a final concentration of 100 mM to early log phase culture of wild-type cells expressing (TMY310) or not expressing (DHT22-1b) *Clb2*–*Myc* in YPD at 28°C, and incubated further. The cell extracts prepared were immunoprecipitated with anti-*Myc* and assayed for histone H1 kinase activity. In parallel, cell extracts were immunoprecipitated with anti-*Myc* antibody, separated by SDS-PAGE and detected with *Clb2*–*Myc*. Activity was normalized relative to the *Clb2* level of each lane. Activity was expressed relative to the activity at time zero (=1.0). (F) Map of the insert ORFs in plasmid pMM2144, which fully complemented the *scz10* mutation. Deletion analysis was carried out to localize the suppressor activity (indicated by the + sign).

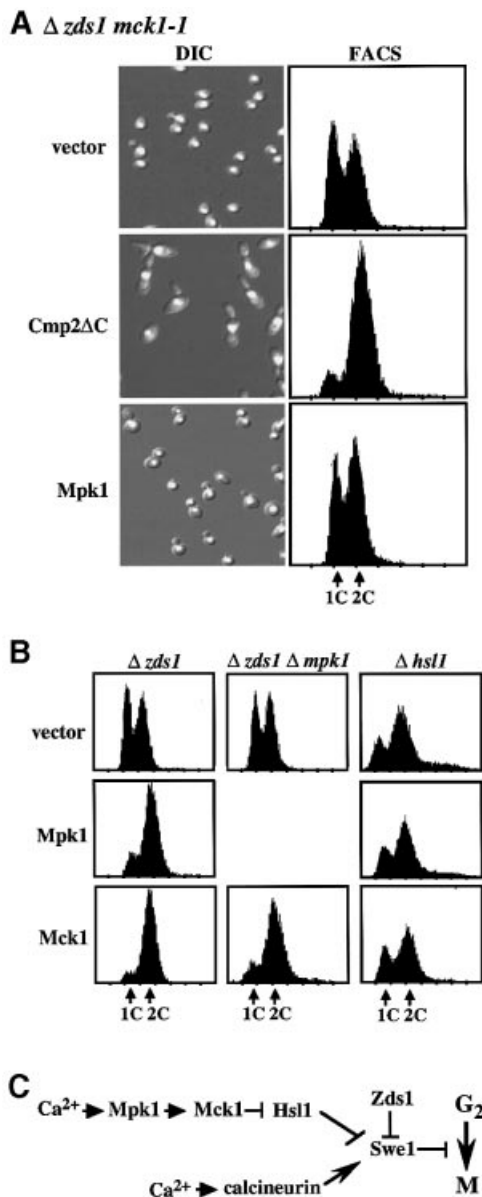


Fig. 2. Mck1 functions downstream of the Mpk1 pathway to induce G₂ delay through the down-regulation of Hsl1. (A) The effect of overexpression of Cmp2ΔC (constitutively active form of the Cmp2 calcineurin catalytic subunit) or Mpk1 in the $\Delta zds1 mck1-1$ strain was examined. The $\Delta zds1 mck1-1$ (YMM2144) strain was transformed with the GAL-regulated plasmids pGAL-Cmp2ΔC (pYES2::Cmp2ΔC), pGAL-Mpk1 (pNV7::Mpk1) or vector alone (pYES2). The transformants were grown in liquid synthetic complete medium (SD minus Ura) at 28°C until early log growth phase. Cells were collected by centrifugation, washed, and suspended in SG (galactose medium, GAL promoter on) and the cells were then incubated at 28°C for 10 h. Cell morphology (DIC), FACS and the DNA content of PI-stained cells were analyzed by flow cytometry analysis. (B) The effect of overexpression of Mpk1 or Mck1 in $\Delta zds1$ (YAT1), $\Delta zds1 \Delta mpk1$ (YMM3) or $\Delta hsl1$ (YMM52) strains was determined. These strains were transformed with pGAL-Mpk1 (pNV7::Mpk1), pGAL-Mck1 (pYES2::Mck1) or vector alone (pYES2). Experimental conditions were as described in (A). (C) Presumed pathway for the regulation of the Hsl1 kinase by Mck1 as suggested by the genetic analyses.

HA-Hsl1 abundance was not altered by the presence of 0.5 μg/ml cycloheximide, suggesting that the regulation is not at the translational level (data not shown). In contrast, HA-Hsl1 in *mck1-1* cells (and *Ampk1* cells, data not

shown) was sustained at higher levels, indicating that the Mpk1–Mck1 pathway was critical in inducing the decrease in the abundance of HA-Hsl1 (Figure 5A). The HA-Hsl1 protein that was present in wild-type cells during incubation with Ca²⁺ migrated more quickly compared with that in *mck1-1* cells. The slower migrating HA-Hsl1 protein in the *mck1-1* cells was verified as a phospho-isoform by treating them with calf intestinal alkaline phosphatase (CIP; data not shown). The preferential disappearance of the phosphorylated species could be explained if the phosphorylated species were degraded or dephosphorylated preferentially prior to the degradation. To examine the second possibility, we asked whether calcineurin was involved in this process. The time course change in HA-Hsl1 abundance in $\Delta cnb1$ cells was slower than that in wild-type cells, and similar to that in *mck1-1* cells (Figure 5A). In addition, the electrophoretic mobility of HA-Hsl1 was similar to that in *mck1-1* cells. These results indicated that the calcineurin-mediated dephosphorylation of Hsl1 occurred prior to its destabilization.

When Cmp2ΔC or Mpk1 was overexpressed in $\Delta zds1$ cells by using a GAL promoter in galactose medium, the HA-Hsl1 level decreased more quickly than when the cells with control plasmid were treated similarly (Figure 5B). In contrast, when Cmp2ΔC was overexpressed in $\Delta zds1 mck1-1$ cells, as well as when Mck1 was overexpressed in $\Delta zds1 \Delta cnb1$ cells, the HA-Hsl1 levels decreased more slowly, confirming that the two pathways coordinately regulate the Hsl1 destabilization (Figure 5B). Basically, similar results were obtained on the wild-type (*ZDS1*) background, except that the rate of Hsl1 decrease was slower than on the $\Delta zds1$ background (data not shown).

Hsl1 may be a phosphorylation substrate of Mck1. To test this possibility, the genes encoding HA-tagged Mck1 or Mck1-1 mutant proteins were constructed and the fused gene was subcloned into a CEN-based plasmid. Western blot analysis following SDS-PAGE of the cell extracts detected a 60 kDa band for HA-Mck1 and a 45 kDa band for HA-Mck1-1, both considerably larger than the predicted molecular masses of the respective proteins (Figure 5C, right). The expression levels of HA-Mck1 and HA-Mck1-1 were similar. The HA-tagged proteins were immunoprecipitated and kinase activity was assayed by incubation with [³²P]ATP using a glutathione S-transferase (GST) fusion of Hsl1(KD) (see below for details) as substrate. A catalytically inactive Hsl1 protein [Hsl1K110A or Hsl1(KD)] was used as substrate, because Hsl1 is autophosphorylatable (Barral *et al.*, 1999). As shown in Figure 5C, Mck1, but not Mck1-1, phosphorylated Hsl1(KD) *in vitro*.

The involvement of calcineurin in Hsl1 dephosphorylation was confirmed by western blot analysis of HA-Hsl1 in the cells grown in the medium containing Ca²⁺. In this experiment, a *cdc4* mutant background was used to protect HA-Hsl1 from degradation (see later). In the cells incubated with Ca²⁺, the broad band was converted to a faster migrating narrow band and this mobility shift was blocked when the cells were incubated in the presence of FK506 (Figure 5D, a). Mobility shift was not induced by Ca²⁺ in $\Delta cnb1$ cells (Figure 5D, a). CIP treatment of HA-Hsl1 present in the wild-type cells incubated with Ca²⁺ in the presence of FK506 showed that the slower

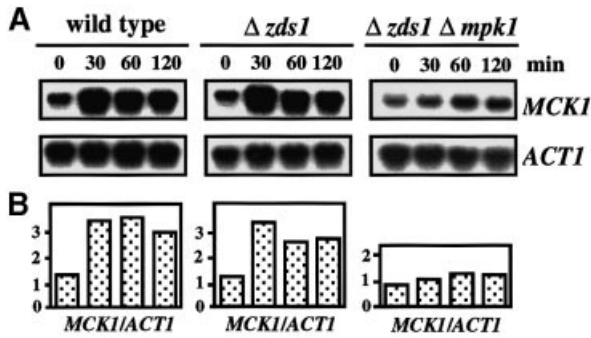


Fig. 3. Mpk1 up-regulates *MCK1* transcription. CaCl₂ was added to a final concentration of 100 mM to early log phase cultures of wild-type (DHT22-1b), $\Delta zds1$ (YAT1) or $\Delta zds1 \Delta mpk1$ (YMM3) cells in YPD at 28°C, and incubated further. RNA samples were extracted at the indicated time and northern blot analysis was performed. The intensity of *MCK1* or *ACT1* mRNA was measured using a BAS-1000 bio-imaging analyzer and the *MCK1* level was normalized to the *ACT1* mRNA level.

migrating species represent a phosphorylated form of Hsl1 (Figure 5D, b). The slow migrating species were not produced with Hsl1(KD), a catalytically inactive mutant protein (see below), indicating that Hsl1 kinase activity is essential to generate the slow migrating species (Figure 5D, a; Barral *et al.*, 1999). These results suggested that Hsl1 is dephosphorylated prior to its degradation, and that calcineurin is responsible for the dephosphorylation *in vivo*.

We next examined whether calcineurin interacts with Hsl1 *in vivo*. Myc-tagged Cnb1 (calcineurin regulatory subunit) was co-precipitated with HA-Hsl1, but not with HA-Hsl1(KD) (see below) (Figure 5E). These results suggested that calcineurin physically interacts with the autophosphorylated, active form of Hsl1, but not with an inactive form.

Ca²⁺-induced Hsl1 destabilization is mediated by the SCF^{Cdc4} complex

Since the Ca²⁺-induced Hsl1 destabilization appeared to occur even at the cell cycle stage(s) when APC activity is low (S, G₂ and early M phases), SCF might be responsible for the regulation of Hsl1 destabilization. To address this possibility, we examined whether the rate of Hsl1 degradation is affected in the mutants *cdc4* and $\Delta grr1$, defective in different F-box proteins of SCF at 37°C, the non-permissive temperature for *cdc4* (Figure 6A). In wild-type and $\Delta zds1$ cells, the Ca²⁺-induced Hsl1 destabilization proceeded more rapidly at 37°C than at 28°C, decreasing to a non-detectable level within 20 min of incubation. Hsl1 in the $\Delta grr1$ cells was degraded rapidly at a rate similar to that in wild-type cells. In contrast, Hsl1 was degraded in *cdc4* cells at a much slower rate, which was similar to that in *mck1-1* or $\Delta cnb1$ cells. These results suggested that the Hsl1 destabilization was dependent on SCF^{Cdc4}, but not SCF^{Grr1}. Consistent with the above result, when Cmp2 Δ C or Mpk1 was overexpressed in *cdc4* mutant cells, the Hsl1 level decreased more slowly than on the wild-type background (data not shown).

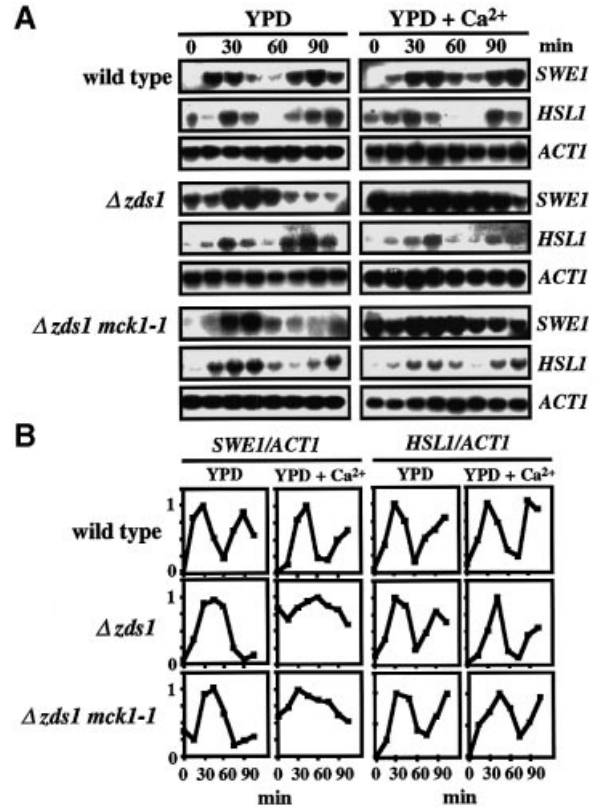


Fig. 4. Mck1 is not involved in the transcriptional regulation of *HSL1*. (A) The oscillation of *SWE1* or *HSL1* mRNA levels during cell cycle progression in various strains that were synchronized with α -factor was determined by northern blotting, using the *SWE1*, *HSL1* and *ACT1* probes. Experimental conditions were as described in the legend to Figure 1C. RNA blot hybridization with the *SWE1*, *HSL1* and *ACT1* probes. (B) Changes in the relative intensity of *SWE1* or *HSL1* mRNA. The amount of *SWE1* or *HSL1* mRNA in (A) was normalized to a constant level of *ACT1* mRNA. In each case, the individual maximum value was referred to as 1. Time 0 represents the point of release from G₁ phase arrest.

Ca²⁺ signal induces Hsl1 delocalization from the bud neck

Hsl1 is localized in the bud neck of cells and physically associates with the bud-side septin ring (Barral *et al.*, 1999; Longtine *et al.*, 2000). We examined whether Hsl1 localization might be affected by the Ca²⁺ signal. To test this possibility, we visualized Hsl1 localization microscopically using an HA-tagged version of Hsl1 expressed from a chromosomally integrated construct (Figure 6C). Strikingly, HA-Hsl1 localized to the neck was delocalized by Ca²⁺ in wild-type and $\Delta zds1$ strains. In contrast, in the Ca²⁺-treated *mck1-1* or $\Delta cnb1$ cells, HA-Hsl1 still remained at the bud neck, suggesting that both Mck1 and calcineurin are required for the delocalization. In *cdc4* cells, the HA signal was delocalized from the bud neck by Ca²⁺ and stayed in the cytoplasm (Figure 6C), suggesting that the delocalization of Hsl1 occurs prior to the degradation.

We next examined whether the Ca²⁺ treatment of the cells may affect septin organization, because the activity of Hsl1, which is co-localized with the septin ring, is dependent on septin function (Barral *et al.*, 1999;

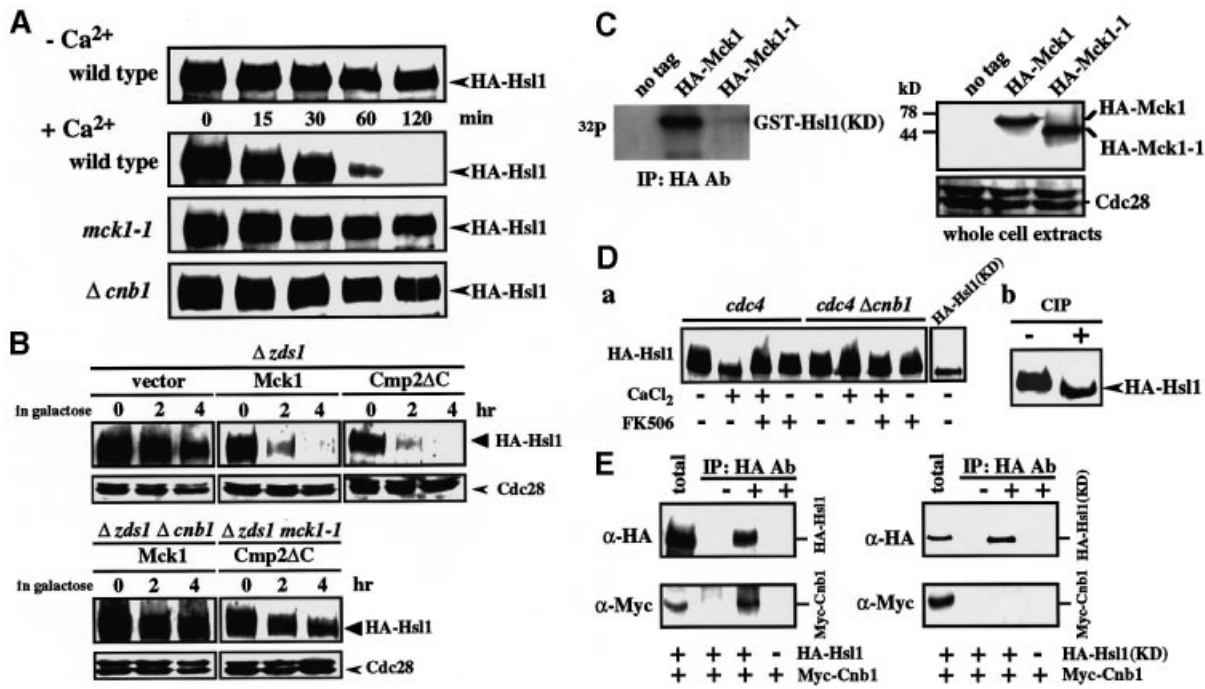


Fig. 5. Mck1 and calcineurin cooperatively regulate Hsl1 abundance. (A) Comparison of Hsl1 abundance in various strains. Wild-type (YMM53), *mck1-1* (YMM71) or $\Delta cnb1$ (YMM72), in which the genomic copy of *HSL1* encodes HA-Hsl1, were grown in liquid YPD at 28°C until early log phase. $CaCl_2$ was added to the cell cultures at the indicated times to a final concentration of 100 mM, samples were taken and western blot analysis was performed. (B) Dependence of Hsl1 destabilization on the functions of the calcineurin and Mpk1–Mck1 pathways. The strain YMM54, in which the genomic copy of *HSL1* was replaced with HA-Hsl1, was transformed with the plasmids pGAL-Cmp2ΔC (pYES2::Cmp2ΔC), pGAL-Mck1 (pYES2::Mck1) or vector alone (pYES2). The transformants were grown in liquid YPR medium containing raffinose at 28°C until early log phase, and then galactose was added. Samples were taken at the indicated times and western blot analysis was performed by probing with anti-HA and anti-Cdc28 (control) antibodies. (C) Phosphorylation of Hsl1 by Mck1 *in vitro*. Cell extracts prepared from $\Delta mck1$ (YMM68) cells expressing HA-Mck1, HA-Mck1-1 or vector alone were immunoprecipitated with anti-HA. Kinase assay was performed by incubating the pellet with [γ - ^{32}P]ATP and recombinant GST-Hsl1(KD). Protein samples were subjected to electrophoresis and exposed for autoradiography. Western blotting was performed to detect HA-Mck1 and HA-Mck1-1, as well as Cdc28 as loading control. (D) (a) Mobility shift of HA-Hsl1 in the cells with activated Ca^{2+} signaling conditions in a calcineurin-dependent manner. Strain *cdc4* (YMM73) or *cdc4 Δcnb1* (YMM74), in which the genomic copy of *HSL1* encodes HA-Hsl1, was incubated at 37°C for 2 h to inactivate *cdc4*. Incubation was carried out for 1 h in the medium with (+) or without (-) $CaCl_2$ (100 mM) or FK506 (1 μg/ml) as indicated. HA-Hsl1 was detected by immunoblotting using anti-HA monoclonal antibody. (b) Phosphatase (CIP) treatment of HA-Hsl1 in the sample prepared similarly to the sample (+ $CaCl_2$, +FK506 of *cdc4* cells) in (a). (E) Physical interaction of Hsl1 and calcineurin. Protein extracts prepared from the cells in which the genomic copy of *HSL1* encodes HA-Hsl1 (YMM67), HA-Hsl1(KD) (YMM62) or none (DHT14) on a $\Delta cnb1$ background harboring plasmid-borne Myc-Cnb1 were immunoprecipitated with anti-HA antibody. The pellets were separated by SDS-PAGE and probed with both anti-HA and anti-Myc antibodies.

Shulewitz *et al.*, 1999; Longtine *et al.*, 2000). In both wild-type and $\Delta zds1$ cells, Ca^{2+} did not cause a significant change in the localization of Cdc11, a component of the septin filament, suggesting that the organization of the septin ring is not affected significantly by Ca^{2+} even on the $\Delta zds1$ background (data not shown). The result suggested that the delocalization of Hsl1 by Ca^{2+} was not due to the perturbation of septin localization.

The localization and the Ca^{2+} -induced delocalization of Hsl1(KD), a catalytically inactive mutant of Hsl1, depend on the presence of wild-type Hsl1

To investigate whether the delocalization and destabilization of Hsl1 are dependent on its kinase activity, we examined the stability of HA-tagged catalytically inactive Hsl1(KD). When the gene encoding HA-Hsl1(KD) was introduced into $\Delta hsl1$ cells, destabilization was not induced by Ca^{2+} . However, when HA-Hsl1(KD) was introduced onto the wild-type *HSL1* background, the destabilization of the mutant protein

was induced (Figure 6B). The presence of a lower level of HA-Hsl1(KD) compared with HA-Hsl1 under the uninduced conditions could be due to the more unstable nature of unlocalized HA-Hsl1(KD). HA-Hsl1(KD) was not localized in the neck on the $\Delta hsl1$ background, suggesting that either the kinase activity or the autophosphorylation on Hsl1 is important for its proper localization (Figure 6B and D). On the wild-type *HSL1* background, in contrast, HA-Hsl1(KD) was localized to the neck in ~30% of the cells (Figure 6D). Since HA-Hsl1(KD) can be phosphorylated by wild-type Hsl1 *in vivo* due to a *trans*-autophosphorylation (Barral *et al.*, 1999), the different behaviors of HA-Hsl1(KD) in the presence and absence of wild-type Hsl1 seem to be a reflection of the phosphorylation state of HA-Hsl1(KD). The data suggested that the Hsl1 phosphorylation, but not the kinase activity itself, is required for Hsl1 localization. It was also suggested that the Hsl1 localization to the bud neck is critically important for the Ca^{2+} -induced Hsl1 destabilization.

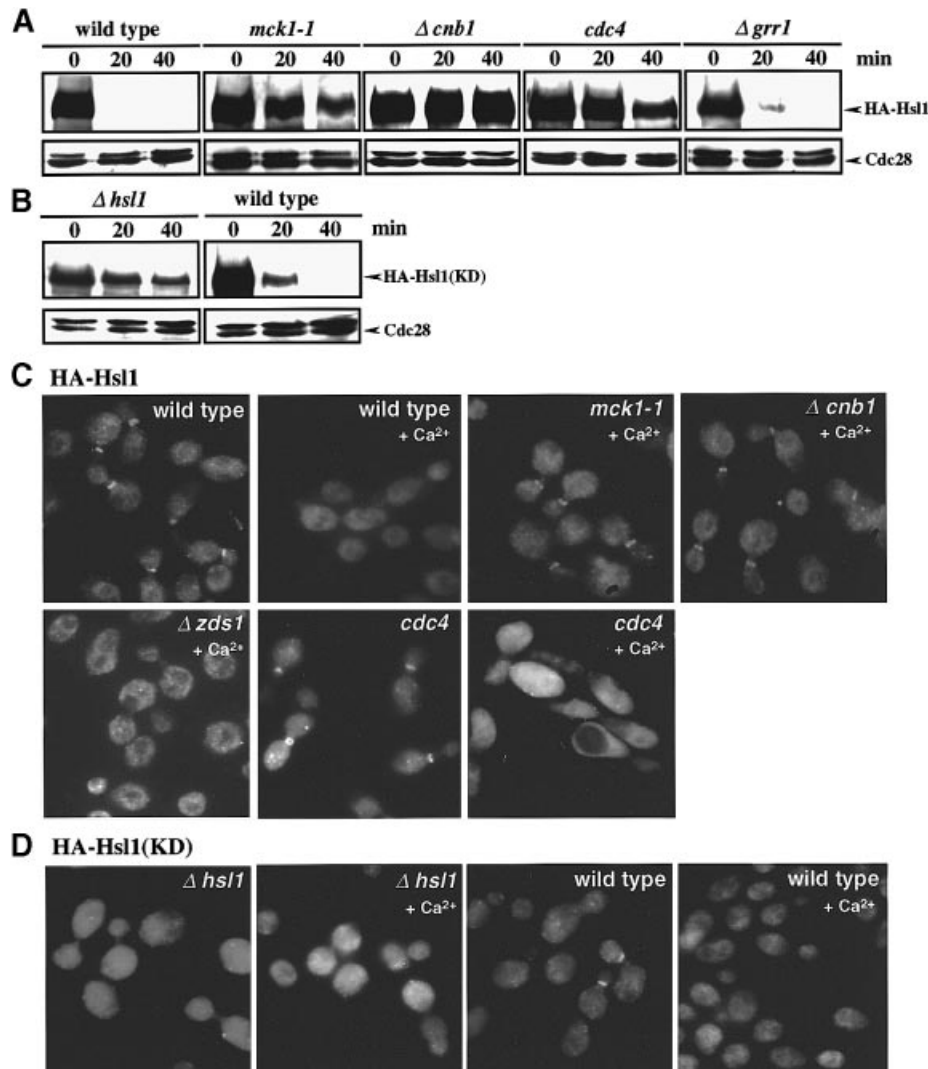


Fig. 6. Ca²⁺ induces SCF^{Cdc4}-mediated Hsl1 destabilization and Hsl1 delocalization from the bud neck in a manner dependent on both calcineurin and Mck1. **(A)** Comparison of Hsl1 abundance in various strains. Wild type (YMM53), *mck1-1* (YMM71), *Δcnb1* (YMM72), *Δzds1* (YMM54), *cdc4* (YMM73) or *Δgrr1* (YMM76), each bearing a gene encoding HA-Hsl1, was grown in liquid YPD at 28°C until early log phase, and then shifted to 37°C for 2 h to inactivate Cdc4. CaCl₂ was added to the cell cultures to a final concentration of 100 mM, samples were taken at intervals of 20 min and western blot analysis was performed. **(B)** Comparison of the Ca²⁺-induced destabilization of a kinase-negative Hsl1, HA-Hsl1(KD), in strains *Δhsl1* (YMM60) or wild-type *HSL1* (YMM66), each bearing a gene encoding HA-Hsl1(KD). Experimental conditions were as described in (A). **(C)** HA-Hsl1 localization in various strains. The cells were grown in liquid YPD at 28°C until early log phase, and then shifted to 37°C for 2 h to inactivate *cdc4*. CaCl₂ was added to the cell cultures to a final concentration of 100 mM, and samples were then taken after 20 min of incubation. Indirect immunofluorescence microscopy was performed as described in Materials and methods. **(D)** Comparison of the localization of HA-Hsl1(KD) in *Δhsl1* (YMM60) or *HSL1* (YMM66) cells. CaCl₂ was added to the cell cultures to a final concentration of 100 mM, and samples were then taken after 20 min of incubation. Experimental conditions were the same as in (C).

Ca²⁺-induced Hsl1 destabilization is independent of the APC pathway

It was demonstrated recently that the Hsl1 level fluctuates during the cell cycle (McMillan *et al.*, 1999b) via an APC-mediated degradation pathway (Burton and Solomon, 2000). We examined whether the Ca²⁺-induced Hsl1 destabilization requires APC function by using an APC mutant *cdc23* (Irniger *et al.*, 1995). As shown in Figure 7A, the Hsl1 destabilization was unaffected in the mutant at the restrictive temperature, indicating that the APC pathway was not significantly involved in the Ca²⁺-induced Hsl1 destabilization.

To examine whether the Ca²⁺-mediated regulation is also important for the cell cycle-dependent Hsl1 fluctu-

ation, we compared the fluctuation patterns in synchronized cell cultures of wild-type and calcineurin-deficient cells expressing HA-Hsl1. As reported previously, the abundance of Hsl1 fluctuated after release from α -factor arrest (Figure 7B and C; McMillan *et al.*, 1999b; Burton and Solomon, 2000). Hsl1 accumulation was periodic during the cell cycle, peaking in G₂ and declining prior to M. A very similar fluctuation pattern was obtained for *Δcnb1* cells, indicating that the calcineurin-dependent degradation pathway did not contribute significantly to the regulation of Hsl1 stability in the normal cell cycle. These results indicated that the Ca²⁺-dependent, SCF^{Cdc4}-mediated pathway and the cell cycle-dependent, APC-mediated pathways are distinct mechanisms for Hsl1 degradation.

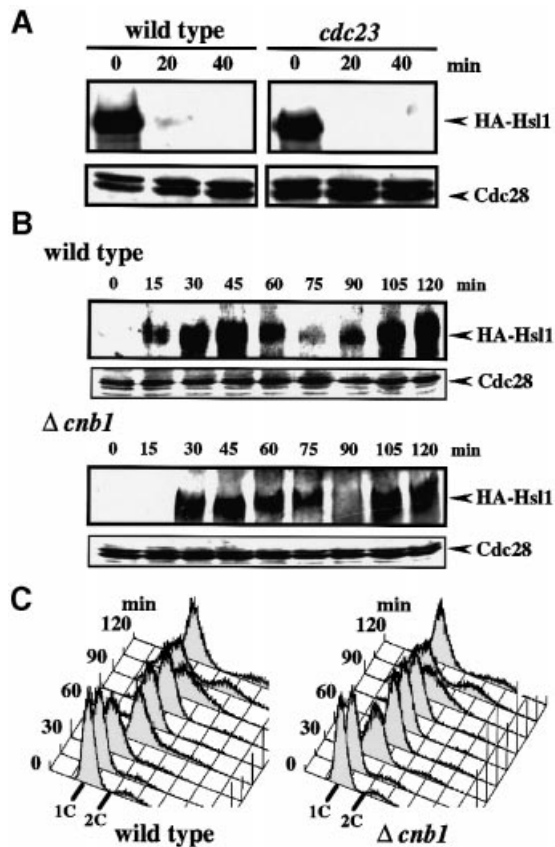


Fig. 7. The Ca^{2+} -regulated Hsl1 destabilization pathway is distinct from the APC pathway. (A) Effect of Ca^{2+} on Hsl1 abundance in an APC mutant. Strains YMM53 or YMM65 (*cdc23*) with integrated HA-Hsl1 were grown in liquid YPD at 28°C until early log phase, and then shifted to 37°C for 2 h to inactivate *cdc23*. CaCl_2 was added to the cell cultures to a final concentration of 100 mM, samples were taken at 20 min intervals and western blot analysis was performed. (B) The fluctuation of the Hsl1 abundance during the cell cycle in a calcineurin-deficient mutant. Strain YMM53 or YMM72 ($\Delta cnb1$), in which the genomic copy of *HSL1* encodes an HA-tagged form of Hsl1, was grown in YPD to early log phase, and cells were synchronized as described in Figure 1C. Samples were taken at 15 min intervals after release in YPD, and whole-cell extracts were prepared. Immunoblotting was performed with antibodies against HA-Hsl1 and Cdc28 as loading control. (C) DNA content of YMM53 or YMM72 cells in a synchronous culture after release from an α -factor-induced G_1 arrest.

Discussion

Identification of Mck1 as the signaling component of the Ca^{2+} -induced G_2 delay

The Ca^{2+} -activated pathways of *S.cerevisiae* induce a delay in the onset of mitosis through the activation of Swe1, a negative regulatory kinase that inhibits the Cdc28–Clb complex. Calcineurin and Mpk1 activate Swe1 at the transcriptional and post-translational level, respectively. In the present study, we identified the *MCK1* gene, which encodes a GSK-3 family protein kinase, as a signaling component downstream of the Mpk1 pathway. The Ca^{2+} -sensitive growth, the Ca^{2+} -induced G_2 delay and polarized bud growth of the $\Delta zds1$ strain were suppressed by *mck1-1* and $\Delta mck1$ mutations. The available genetic, biochemical and imaging data are consistent with a novel signaling pathway in which Mck1 and calcineurin are

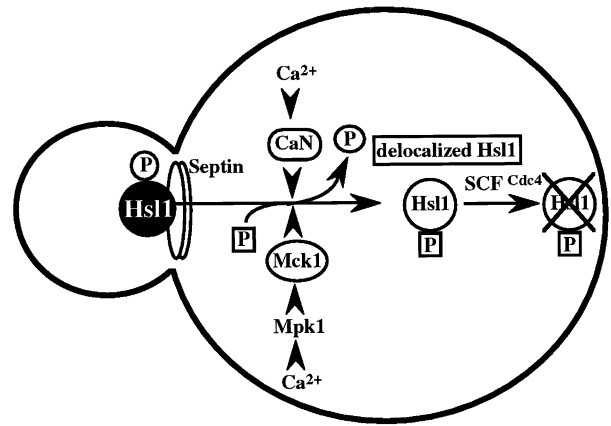


Fig. 8. Model for the role of the Ca^{2+} signaling pathways of budding yeast in regulating the G_2 phase delay through the delocalization and destabilization of Hsl1 from the bud neck. CaN, calcineurin.

activated by Ca^{2+} , which in turn coordinately down-regulate Hsl1 by inducing the delocalization from the bud neck and subsequent destabilization via the SCF^{Cdc4}-mediated pathway (Figure 8). Therefore, calcineurin is involved in the regulation of the G_2/M cell cycle progression by Swe1 activation at two distinct steps, at the transcriptional and post-transcriptional levels through the down-regulation of the Swe1 inhibitor Hsl1 by dephosphorylating it (via delocalization and destabilization). A high Ca^{2+} concentration was able to induce the G_2 delay even in $\Delta hsl1$ cells, and the G_2 delay was abolished by the deletion of either *SWE1* or *CNB1* (data not shown), indicating that the Ca^{2+} -induced G_2 delay in $\Delta hsl1$ cells is due mainly to the calcineurin-dependent transcriptional activation of *SWE1*. However, there still remains a possibility that the Ca^{2+} signaling pathways negatively regulate Mih1, the phosphatase that reverses the Cdc28 phosphorylation catalyzed by Swe1.

Because various phenotypes displayed by the *mck1-1* allele were more severe than those of the null allele (Figure 1A), we characterized the *mck1-1* mutation. A catalytically inactive, truncated Mck1-1 protein was produced at a level comparable to that of wild-type Mck1 (Figure 5C). The yeast genome encodes three additional GSK-3 family proteins, Mds1/Rim11, Mrk1 and Yol128c, which may share redundant functions with Mck1 (Andoh *et al.*, 2000). The more potent phenotypes exhibited by the *mck1-1* allele compared with the $\Delta mck1$ allele would be explained if the functional Mck1 homologs were excluded by the Mck1-1 protein from the presumed Mck1 complexes.

Some physiological roles were described previously for Mck1. Mck1 is important in mitotic chromosome segregation specific to CDEIII function (Shero and Hieter, 1991). In addition, the Mck1 function is required in diploid cells for expression of *IME1*, a gene encoding an early meiotic activator, and subsequent ascus maturation (Neugeborn and Mitchell, 1991). In these Mck1-mediated processes, however, the upstream and downstream regulatory mechanisms are not yet known. In addition to these, we have identified a novel event in which Mck1 plays a key regulatory role in inducing the cell cycle delay by Ca^{2+} . Thus, Mck1 kinase seems to function in a variety of processes that operate in apparently unrelated intracellular

events. Whether Mck1 has multiple target proteins in each of these events or a common target protein is responsible for all of them remains unclear. However, given that the Hsl1 kinase is a potential target of Mck1, it seems unlikely that Hsl1 is also responsible for the activation of the sporulation pathways. In fact, a $\Delta hsl1/\Delta hsl1$ diploid could sporulate normally, showing that Hsl1 may not be important in this event (data not shown). However, it still remains to be seen whether Hsl1 is also involved in the mitotic chromosome segregation specific to CDEIII function. The astonishingly diverse functions of Mck1 could be rationalized if the Mck1 kinase, upon activation by signals, induces destabilization of a key regulatory protein of the respective event through a ubiquitylation pathway, in a manner analogous to the mechanism described for Hsl1. The involvement of the GSK-3 family protein kinase in targeting the key regulatory protein for destabilization seems to be conserved in eukaryotes, as will be discussed for β -catenin.

An elaborate genetic screen using a $\Delta zds1 \Delta zds2$ strain that displays a strong mitotic defect due to a severe morphogenic defect was conducted recently by another group to isolate mutants with a defect in the signaling pathways for the morphogenesis checkpoint (McMillan *et al.*, 1999a). In that study, the screening gave rise to *SWE1* gene mutants and a dominant checkpoint defective allele of *CDC28*, but not to any of the mutant loci of the Ca²⁺ signaling pathways. This result suggested that the Ca²⁺ signal may not be responsible for the morphogenesis checkpoint (Lew and Reed, 1995; Sia *et al.*, 1996; McMillan *et al.*, 1998). It is still possible, however, that *Zds2* protein may have some unknown function, in addition to its functional redundancy with *Zds1*.

Roles of Mck1 and calcineurin in regulating Hsl1 delocalization and destabilization

Our data demonstrated that the Hsl1 protein was destabilized via the SCF^{Cdc4} pathway in a manner dependent on Ca²⁺ signaling. The physiological consequences caused by Ca²⁺ can be explained by the decreased abundance of Hsl1. Surprisingly, both calcineurin-deficient cells and *mck1-1* cells displayed apparently similar defects in the delocalization and destabilization of Hsl1, suggesting that these events are rigorously regulated by the combined actions of calcineurin and Mck1 (Figure 8). Considering that the delocalized Hsl1 remains in the cytosol of *cdc4* cells, Hsl1 delocalization may be viewed as a primary event that is regulated by calcineurin and Mck1, and it seems important for destabilization via the SCF^{Cdc4} pathway. This view was supported further by the observation that Hsl1(KD), which was unable to localize to the bud neck, was a substrate of SCF^{Cdc4} only when the mutant protein was localized to the neck with the aid of wild-type Hsl1 (Figure 6B and D).

The observations that phospho-Hsl1 is a calcineurin substrate and calcineurin co-immunoprecipitates with Hsl1 suggested that Hsl1 and calcineurin interact *in vivo*. We also demonstrated that Mck1 phosphorylates Hsl1 *in vitro*. In fact, Hsl1 contains as many as 54 potential phosphorylation sites (S/TXXXS/T) known as the consensus sequence for a GSK-3 β phosphorylation site (Plyte *et al.*, 1992). It still remains to be clarified whether Hsl1 phosphorylation by Mck1 requires prior phosphorylation

by other protein kinases for the delocalization and destabilization of Hsl1. It is known, in some cases, that the substrate recognition by GSK-3 β requires prior phosphorylation by a priming kinase. With other proteins, however, such as glycogen synthase, inhibitor-2, adenomatous polyposis coli and rAxin, prior phosphorylation is not required (Plyte *et al.*, 1992; Ikeda *et al.*, 1998).

Because Hsl1 is associated with the septin filament together with various other components including Hsl7 and Swe1 (Barral *et al.*, 1999; Shulewitz *et al.*, 1999; Longtine *et al.*, 2000), the mechanism underlying Hsl1 delocalization that involves phosphorylation and dephosphorylation of Hsl1 seems very complex and still remains to be solved.

Conserved function of GSK-3 family kinases in inducing protein destabilization

In SCF^{Cdc4}-dependent Hsl1 destabilization, Mck1 appeared to be important for targeting Hsl1 for ubiquitylation and proteolysis by the proteasome. This scheme is reminiscent of the mechanism for β -catenin destabilization in embryos (Aberle *et al.*, 1997). β -catenin in Wnt signaling depends on post-translational modifications and its interactions with protein partners localized in the cytoplasm and nucleus (for a review see Miller and Moon, 1996). A complex containing axin that directly associates with β -catenin, GSK-3 β and adenomatous polyposis coli was implicated in negatively regulating the Wnt signaling pathway. The stability of β -catenin is controlled by phosphorylation, most probably by GSK-3 β , which targets β -catenin to the ubiquitin-proteasome pathway (Aberle *et al.*, 1997). Wnt signaling regulates interactions between axin and β -catenin and the subsequent stabilization of β -catenin. In the absence of the Wnt signal, GSK-3 β targets β -catenin for a degradative pathway. Signaling by Wnt inactivates GSK-3 β , and thus stabilizes β -catenin and increases availability for signaling. Thus, GSK-3 kinase regulation of protein stabilization or destabilization in response to a signal seems to be an evolutionarily conserved mechanism for regulating protein abundance through phosphorylation, ubiquitylation and proteolysis by the proteasome.

Hsl1 is a common substrate of SCF- and APC-mediated degradation depending on the signals

It was demonstrated recently that the Hsl1 level oscillates in a cell cycle-dependent manner, being absent in late M to G₁, when APC activity is high, and present in S, G₂ and early M phase, when APC activity is low (Burton and Solomon, 2000). The inactivation of Hsl1 by the APC-mediated degradation in late mitosis may be important to allow Swe1 to perform its role in the morphogenesis pathway in the G₁ and S phases. The Ca²⁺ signal induces a G₂ cell cycle delay through the activation of Swe1, responding to a stress that causes a membrane stretch (Mizunuma *et al.*, 1998). Thus, the SCF-mediated Hsl1 destabilization may be viewed as a mechanism that serves to activate Swe1 rapidly through the degradation of Hsl1 in response to a stress at the cell cycle stages when the APC activity is low (S, G₂ and early M phases).

Table I. Yeast strains used in this study

Strain	Genotype	Source or reference
W303 derivatives		
DHT22-1b	<i>MATa trp1 leu2 ade2 ura3 his3 can1-100</i>	W303-1A from Dr Rothstein
YAT1	<i>MATa Δzds1::TRP1</i>	Mizunuma <i>et al.</i> (1998)
DHT14	<i>MATa Δcnb1::HIS3</i>	Nakamura <i>et al.</i> (1993)
TNP46	<i>MATa Δmpk1::HIS3</i>	Nakamura <i>et al.</i> (1996)
YMM3	<i>MATa Δzds1:TRP1 Δmpk1::HIS3</i>	Mizunuma <i>et al.</i> (1998)
YMM5-8c	<i>MATa Δswe1::HIS3</i>	Mizunuma <i>et al.</i> (1998)
YMM22	<i>MATa mck1-1</i>	this study
YMM36	<i>MATa Δzds1:TRP1 mck1 YIp-URA3-MCK1</i> integrated at <i>MCK1</i>	this study
YMM51	<i>MATa Δhsl1::URA3 (hsl1Δ2::URA3)</i>	Ma <i>et al.</i> (1996)
YMM52	<i>MATa Δhsl1::ura3</i>	this study
YMM53	<i>MATa Δhsl1::ura3 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM54	<i>MATa Δhsl1::ura3 Δzds1::TRP1 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM59	<i>MATa Δhsl1::ura3 cdc4 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM60	<i>MATa Δhsl1::ura3 3HA-HSL1(KD)</i> integrated at <i>HSL1</i>	this study
YMM62	<i>MATa Δhsl1::ura3 Δcnb1::HIS3 3HA-HSL1(KD)</i> integrated at <i>HSL1</i>	this study
YMM65	<i>MATa Δhsl1::ura3 cdc23 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM66	<i>MATa 3HA-HSL1(KD)</i> integrated at <i>HSL1</i>	this study
YMM67	<i>MATa Δhsl1::ura3 Δcnb1::HIS3 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM68	<i>MATa Δmck1::TRP1</i>	this study
YMM69	<i>MATa Δmck1::TRP1 Δzds1::TRP1</i>	this study
YMM71	<i>MATa Δhsl1::ura3 mck1-1 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM72	<i>MATa Δhsl1::ura3 Δcnb1::HIS3 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM73	<i>MATa Δhsl1::ura3 cdc4 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM74	<i>MATa Δhsl1::ura3 Δcnb1::HIS3 cdc4 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
YMM76	<i>MATa Δgrr1::LEU2 3HA-HSL1</i> integrated at <i>HSL1</i>	this study
W1950-8c	<i>MATα cdc4 HIS3</i>	A.Toh-e
TMY307	<i>MATa cdc23</i>	A.Toh-e
TMY310	<i>MATa Δbar1::HIS3 Clb2-Myc12-URA3</i>	A.Toh-e
TK-61	<i>MATa Δgrr1::LEU2</i>	T.Kishi
YMM2144	<i>MATa Δzds1:TRP1 mck1-1</i>	this study

Materials and methods

Strains, media and genetic methods

Yeast strains used in this study are listed in Table I. The mutant strains were all derivatives of W303-1A. Media used were as described previously (Mizunuma *et al.*, 1998). Disruption of the *HSL1* gene was performed using a *hsl1* disruption plasmid *Δhsl1 NS::URA3* (a gift from M.Grunstein; Ma *et al.*, 1996). The yeast *mck1::TRP1* mutant was constructed by introducing *PvuII*-digested pUC119-mck1::TRP1 plasmid into diploid *trp1* cells.

Plasmids

The plasmids used for creating the *mck1* disruption alleles were as follows: the *HindIII*–*SphI* *MCK1* fragment (pMM2144ΔSphI) was subcloned into pUC119, and the resulting plasmid was cut with *NcoI*, filled in with Klenow enzyme and ligated with *XhoI* linker (pUC119-mck1::XhoI). Finally, the *SacII*–*XhoI* *TRP1* fragment was ligated to *SacII*–*XhoI*-digested pUC119-mck1::XhoI to create plasmid pUC119-mck1::TRP1 plasmid. The plasmid pYES2-Mck1 was constructed to express the *MCK1* gene under the control of the *GAL1* promoter. The plasmid YIp5-Mck1 was constructed to establish allelism between *SCZ10* and *MCK1*. The plasmid pRS315-HA-Mck1 was constructed to express full-length *MCK1* with three copies of the HA epitope at its N-terminus. The chromosomal *HSL1* gene was replaced with a gene encoding Hs11 with a triple HA epitope tag at its N-terminus and expressed from its own promoter, using plasmid YIp5-HA-Hs11 (YIp5, an integrating vector containing *LEU2⁺* as a selectable marker). Construction of the inactive kinase allele Hs11(KD) was carried out basically as described (Barral *et al.*, 1999). The plasmid YIp5-HA-Hs11(KD) was constructed to integrate Hs11(KD) with a triple HA epitope at its N-terminus. The plasmid pGEX-KG-Hs11(KD) was constructed to express GST fused to Hs11(KD) at its N-terminus. The plasmid YCplac22-9Myc-CNB1 was constructed to express full-length *CNB1* with a Myc epitope at its C-terminus.

Flow cytometry and cell microscopy

Flow cytometric analysis was carried out as described previously (Mizunuma *et al.*, 1998). Yeast cells were fixed with formaldehyde to a

final concentration of 3.7%, and viewed by differential interference contrast (DIC) microscopy. To visualize nuclei, cells were stained with DAPI (4',6-diamidino-2-phenylindole).

Cell culture synchronization, RNA isolation and northern blot analysis

Cell culture synchronization and northern blot analysis were carried out essentially as described previously (Ma *et al.*, 1996; Mizunuma *et al.*, 1998).

Preparation of extracts, immunoprecipitations and kinase assays

Preparation of cell extracts and immunoprecipitations were carried out essentially as described previously (Barral *et al.*, 1999). For detection of the HA-tagged proteins, Myc-tagged Cnb1 or Cdc28, monoclonal antibodies 12CA5 to the HA epitope, 9E10 to the Myc epitope or anti-PSTAIR, respectively, were used.

In Hs11 dephosphorylation reactions, HA-Hs11 was immunoprecipitated. CIP was added to the beads containing the HA-Hs11 immune complex and incubated at 37°C for 30 min.

Clb2 kinase and Mck1 kinase activity were assayed as described (Barral *et al.*, 1999) using histone H1 or GST–Hs11(KD) as substrate, respectively.

Indirect immunofluorescence

Indirect immunofluorescence microscopy was performed as described (Pringle *et al.*, 1991).

Acknowledgements

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