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The *Saccharomyces cerevisiae* 14-3-3 Proteins Are Required for the G_1/S Transition, Actin Cytoskeleton Organization and Cell Wall Integrity

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ABSTRACT

14-3-3 proteins are highly conserved polypeptides that participate in many biological processes by binding phosphorylated target proteins. The *Saccharomyces cerevisiae BMH1* and *BMH2* genes, whose concomitant deletion is lethal, encode two functionally redundant 14-3-3 isoforms. To gain insights into the essential function(s) shared by these proteins, we searched for high-dosage suppressors of the growth defects of temperature-sensitive *bmh* mutants. Both the protein kinase C1 (Pkc1) and its upstream regulators Wsc2 and Mid2 were found to act as high dosage suppressors of *bmh* mutants' temperature sensitivity, indicating a functional interaction between 14-3-3 and Pkc1. Consistent with a role of 14-3-3 proteins in Pkc1-dependent cellular processes, shift to the restrictive temperature of *bmh* mutants severely impaired initiation of DNA replication, polarization of the actin cytoskeleton, and budding, as well as cell wall integrity. Because Pkc1 acts in concert with the Swi4-Swi6 (SBF) transcriptional activator to control all these processes, the defective G_1/S transition of *bmh* mutants might be linked to impaired SBF activity. Indeed, the levels of the G_1 cyclin *CLN2* transcripts, which are positively regulated by SBF, were dramatically reduced in *bmh* mutants. Remarkably, budding and DNA replication defects of *bmh* mutants were suppressed by *CLN2* expression from an SBF-independent promoter, suggesting that 14-3-3 proteins might contribute to regulating the late G_1 transcriptional program.

THE 14-3-3 proteins are a large family of highly conserved, ubiquitously expressed acidic polypeptides of 28–33 kDa found in all eukaryotes. At least 7 isoforms are present in mammals and up to 15 isoforms are present in plants, while 2 isoforms have been identified in yeast, *Drosophila melanogaster*, and *Caenorhabditis elegans* (reviewed in Hermeking 2003; Dougherty and Morrison 2004). They form homo- and heterodimers able to bind protein ligands that are usually phosphorylated on serine or threonine residues of consensus binding motifs (Jones *et al.* 1995; Muslin *et al.* 1996; Yaffe *et al.* 1997; Chaudhri *et al.* 2003). By inducing conformational changes or steric hindrance in protein ligands, 14-3-3 proteins can activate/repress their enzymatic activity, prevent their degradation, modulate their localization, and/or facilitate/inhibit their modifications and interactions (reviewed in Hermeking 2003; Dougherty and Morrison 2004). Targets of 14-3-3 family members are found in all subcellular compartments and include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, checkpoint and apoptosis factors, and tumor suppressors. This plethora of interacting proteins allows 14-3-3 to play important roles in a wide range of regulatory processes such as cell cycle control, mitogenic signal transduction, and apoptotic cell death and to be implicated in carcinogenesis and some human diseases (reviewed in Dougherty and Morrison 2004). However, because multiple 14-3-3 isoforms are present in mammals and 14-3-3 proteins have several binding targets, the mechanisms underlying 14-3-3 functions are not fully understood.

The two *Saccharomyces cerevisiae* members of the 14-3-3 family, sharing 93% amino acid identity, are encoded by the *BMH1* and *BMH2* genes. While single *bmh1*Δ and *bmh2*Δ mutants do not show detectable growth defects compared to wild type, the *bmh1 bmh2* double disruption is lethal in most laboratory strains (van Heusden *et al.* 1992, 1995; Gelperin *et al.* 1995; Roberts *et al.* 1997).

Although their essential functions are not well understood, budding yeast Bmh proteins appear to be involved in many cellular processes. For example, they modulate the activity of several transcription factors. In fact, loss-of-function mutations impairing the *SIN4* or the *RTG3* genes, encoding a global transcriptional regulator and a basic helix–loop–helix transcription factor, respectively, suppress the temperature-sensitive phenotype of a *bmh1 bmh2* mutant (van Heusden and Steensma 2001). Moreover, Bmh1 physically interacts with phosphorylated Rtg3, suggesting that 14-3-3 proteins inhibit Rtg3 transcriptional activation function by binding its phosphorylated form (van Heusden and

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Steensma 2001). Finally, Bmh1 physically interacts with Msn2 and Msn4, two transcription factors required to activate a large number of stress-related genes, and retains their phosphorylated forms in the cytoplasm (Beck and Hall 1999).

Vesicular transport and cortical actin network organization also likely involve 14-3-3 proteins (Gelperin *et al.* 1995; Roth *et al.* 1999). In fact, *S. cerevisiae* cells overproducing the carboxy-terminal region of Bmh2 fail to polarize vesicular transport and show a disrupted actin cytoskeleton (Roth *et al.* 1999). Moreover, 14-3-3 proteins interact with many proteins involved in cytoskeletal regulation in both yeast and mammals (Jin *et al.* 2004). In particular, two-hybrid interactions have been reported for Bmh2 with Msb3 (Mayordomo and Sanz 2002), which is involved in actin cytoskeleton organization (Bach *et al.* 2000; Bi *et al.* 2000), and with Gic2 (Mayordomo and Sanz 2002), which is required together with Gic1 for cytoskeleton polarization during bud emergence (Brown *et al.* 1997; Chen *et al.* 1997). Both Bmh1 and Bmh2 interact also with the p21-activated kinase (PAK) Ste20. This interaction appears to be specifically required for Ras/MAPK cascade signaling during pseudohyphal development (Roberts *et al.* 1997). Finally, mammalian 14-3-3 proteins regulate actin dynamics by stabilizing phosphorylated cofilin, a family of proteins essential for high rates of actin filament turnover through regulation of the actin polymerization/depolymerization cycles (Gohla and Bokoch 2002). By interacting with various regulatory proteins, 14-3-3 proteins participate in diverse signal-transduction pathways. In fact, cell lethality caused by Bmh depletion can be suppressed by hyperactivating the Ras/cAMP-dependent protein kinase A (PKA) pathway through overproduction of Tpk1, the PKA catalytic subunit (Gelperin *et al.* 1995). Consistent with a link between 14-3-3 and PKA, Bmh proteins are dispensable for yeast cell viability in Σ1278b background (Roberts *et al.* 1997), where the Ras/cAMP signaling pathway is hyperactivated (Stanhill *et al.* 1999). However, *bmh1*Δ *bmh2*Δ Σ1278b derivative cells exhibit osmoremediable temperature sensitivity and sensitivity to high osmolarity (Roberts *et al.* 1997), suggesting that some functions of Bmh proteins are still required at 37° even in this background. Moreover, 14-3-3 proteins have been implicated also in Ras/MAPK cascade signaling in vertebrates (Fantl *et al.* 1994; Li *et al.* 1995) and during pseudohyphal development in *S. cerevisiae* (Roberts *et al.* 1997). Finally, vertebrate 14-3-3 proteins were shown to inhibit or activate protein kinase C (PKC), which is involved in many signaling processes (Toker *et al.* 1990; Isobe *et al.* 1992; Tanji *et al.* 1994), and to stimulate the interaction between PKC and the mitogen-stimulated Raf1 kinase that controls cell growth (Van Der Hoeven *et al.* 2000).

In a previous study we isolated four *bmh1* alleles, whose presence in the cell as the sole 14-3-3 source caused temperature-sensitive growth (Lottersberger *et al.* 2003). To provide new insights into the essential functions of the *S. cerevisiae* 14-3-3 proteins, we carried out a detailed phenotypic characterization of these mutants and searched for high-dosage suppressors of their temperature sensitivity. We provide evidence that hyperactivation of the protein kinase C1 (Pkc1)-dependent pathways suppresses the growth defects of these *bmh* mutants, suggesting that 14-3-3 proteins functionally interact with Pkc1. Accordingly, *bmh* mutants are impaired in Pkc1-regulated processes at the G_1/S transition, such as budding, initiation of DNA replication, actin cytoskeleton polarization, and cell wall integrity. Our data suggest that both the temperature sensitivity and the G_1/S transition defects of our *bmh* mutants might be ascribed to an impaired activity of the Swi6/Swi4 (SBF) transcription factor, which is known to act in concert with Pkc1 to control all the processes described above.

MATERIALS AND METHODS

Yeast strains and media: The relevant genotypes of all the yeast strains are listed in Table 1. All the strains used during this study were derivatives of W303 (*MAT***a** or *MAT*α, *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ssd1*).

Strains YLL1082, YLL1081, YLL1120, and YLL1092 were previously described (Lottersberger *et al.* 2003). Wild-type, *bmh1-103 bmh2*Δ, *bmh1-221 bmh2*Δ, *bmh1-266 bmh2*Δ, and *bmh1-342 bmh2*Δ strains carrying either the 2μ vector or 2μ *WSC2* or 2μ *MID2* or 2μ *GIC1* plasmids were constructed by transforming strains W303, YLL1082, YLL1081, YLL1120, and YLL1092 with plasmids YEplac195 (2μ *URA3*), pML489 (2μ *WSC2 URA3*), pML490 (2μ *MID2 URA3*), and pML493 (2μ *GIC1 URA3*), respectively.

A *MAT*α strain, carrying the *GAL-CLN2* construct integrated at the *CLN2* chromosomal locus and obtained after sporulation of the diploid L96 kindly provided by L. Dirick (Montpellier, France), was crossed to strains YLL1082, YLL1081, YLL1120, and YLL1092 to obtain DMP4370/2D, DMP4372/3B, DMP4373/7C, and DMP4465/3B strains, respectively. Strain DMP4357/1B was obtained after sporulation of the diploid L96. Strain YLL1906, carrying the deletion of the *PKC1* gene, was kindly provided by R. Tisi (University of Milano-Bicocca, Italy).

Strains DMP4436/3C, DMP4439/6B, DMP4440/5C, and DMP4441/5A were meiotic segregants from crosses of strains YLL1082, YLL1081, YLL1120, and YLL1092, respectively, with a *MAT*α *cdc24-1* strain. Strains DMP4430/7A, DMP4433/10B, DMP4434/7B, and DMP4435/4B strains were meiotic segregants from crosses of strains YLL1082, YLL1081, YLL1120, and YLL1092, respectively, with a *MAT*α *cdc42-1* strain.

The accuracy of all gene replacements and integrations was verified by Southern blot analysis or PCR. Standard yeast genetic techniques and media were according to Rose *et al.* (1990). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 1% galactose (YEP+raf+gal). Transformants carrying the *KANMX4* cassette were selected on YEPD plates containing 400 μg/ml G418 (US Biological).

Plasmids: To obtain plasmid pML490, containing a *MID2* fragment spanning from 663 bp upstream of the coding region start codon to 425 bp downstream of the stop codon,

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Relevant genotype	Reference/Source
YLL1081	*MAT***a** *bmh2Δ::KanMX4 bmh1-221::LEU2::bmh1Δ::HIS3*	Lottersberger *et al.* (2003)
YLL1082	*MAT***a** *bmh2Δ::KanMX4 bmh1-103::LEU2: bmh1Δ::HIS3*	Lottersberger *et al.* (2003)
YLL1092	*MAT***a** *bmh2Δ::KanMX4 bmh1-342::LEU2: bmh1Δ::HIS3*	Lottersberger *et al.* (2003)
YLL1120	*MAT***a** *bmh2Δ::KanMX4 bmh1-266::LEU2: bmh1Δ::HIS3*	Lottersberger *et al.* (2003)
DMP4436/3C	*MAT***a** *bmh2Δ::KanMX4 bmh1-103::LEU2::bmh1Δ::HIS3 cdc24-1*	This study
DMP4430/7A	*MAT***a** *bmh2Δ::KanMX4 bmh1-103::LEU2::bmh1Δ::HIS3 cdc42-1*	This study
DMP4439/6B	*MAT***a** *bmh2Δ::KanMX4 bmh1-221::LEU2::bmh1Δ::HIS3 cdc24-1*	This study
DMP4433/10B	*MAT***a** *bmh2Δ::KanMX4 bmh1-221::LEU2::bmh1Δ::HIS3 cdc42-1*	This study
DMP4440/5C	*MAT***a** *bmh2Δ::KanMX4 bmh1-266::LEU2::bmh1Δ::HIS3 cdc24-1*	This study
DMP4434/7B	*MAT***a** *bmh2Δ::KanMX4 bmh1-266::LEU2::bmh1Δ::HIS3 cdc42-1*	This study
DMP4441/5A	*MAT***a** *bmh2Δ::KanMX4 bmh1-342::LEU2::bmh1Δ::HIS3 cdc24-1*	This study
DMP4435/4B	*MAT***a** *bmh2Δ::KanMX4 bmh1-342::LEU2::bmh1Δ::HIS3 cdc42-1*	This study
L96	*MAT***a**/α *CLN2/cln2::GAL-CLN2::URA3*	L. Dirick
DMP4357/1B	*MAT***a** *cln2::GAL-CLN2::URA3*	This study
DMP4370/2D	*MAT***a** *bmh2Δ::KanMX4 bmh1-103::LEU2::bmh1Δ::HIS3 cln2::GAL-CLN2::URA3*	This study
DMP4372/3B	*MAT***a** *bmh2Δ::KanMX4 bmh1-221::LEU2::bmh1Δ::HIS3 cln2::GAL-CLN2::URA3*	This study
DMP4373/7C	*MAT***a** *bmh2Δ::KanMX4 bmh1-266::LEU2::bmh1Δ::HIS3 cln2::GAL-CLN2::URA3*	This study
DMP4465/3B	*MAT***a** *bmh2Δ::KanMX4 bmh1-342::LEU2::bmh1Δ::HIS3 cln2::GAL-CLN2::URA3*	This study

a 2219 bp *MID2* fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP551 (5′-CGG GAT CCC GAT TGA GAG ATC TCA CGG AAA TG-3′) and PRP552 (5′-CGG GAT CCC GTC ACA GAA CTC GGT AAG TTT TC-3′) as primers. The PCR amplification product was then cloned into the *Bam*HI site of plasmid YEplac195 (Gietz and Sugino 1988).

To obtain plasmid pML489, containing the *WSC2* ORF flanked by 459 bp upstream of the start codon and 293 bp downstream of the stop codon, a 2264 bp *WSC2* fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP543 (5′-CGG GAT CCC GCT ACG GTA AAC ATG CCT GAT GG-3′) and PRP544 (5′-CGG GAT CCC GTG TGA TCT AGC ACT TCT CCC AG-3′) as primers. The PCR amplification product was then cloned into the *Bam*HI site of plasmid YEplac195.

To obtain plasmid pML493, containing the *GIC1* ORF flanked by 343 bp upstream of the start codon and 282 bp downstream of the stop codon, a 1570-bp *GIC1* fragment was amplified by PCR using yeast genomic DNA as template and the oligonucleotides PRP561 (5′-GGG GTA CCC CGT TGT CTG AGC AGG AAT AAA GAG-3′) and PRP562 (5′-GGG GTA CCC CGG GTA GTA GAC ATC GCT ATT ATC-3′) as primers. The PCR amplification product was then cloned into the *Kpn*I site of plasmid YEplac195.

Plasmid YEplac112 (Gietz and Sugino 1988), carrying the *PKC1* gene, was kindly provided by R. Tisi (University of Milano-Bicocca, Italy).

Search for high-dosage suppressors: To search for high-dosage suppressors of the temperature sensitivity caused by the *bmh1-266* mutation, strain YLL1120 was transformed with an *S. cerevisiae* genomic library on the basis of the multicopy 2μ vector YEp24 (Carlson and Botstein 1982). Ura^+ transformants were tested for their ability to grow at 37° on YEPD plates, which inhibited the untransformed strain. Plasmids from transformants showing cosegregation of the thermoresistance with the *URA3* vector marker were recovered and introduced again into the YLL1120 strain, to confirm their ability to suppress *bmh1-266* temperature sensitivity. Restriction analysis allowed us to identify several classes of plasmids containing different yeast genomic fragments. The nucleotide sequences of both ends of the smallest DNA insert of each plasmid class were determined and compared with the whole *S. cerevisiae* genomic sequence in the Saccharomyces Genome Database. Since most inserts contained several ORFs, the suppressor genes were identified by cloning subfragments of the inserts into the 2μ plasmid YEplac195 and testing the derivative plasmids for their ability to suppress the temperature sensitivity of the *bmh1-266 bmh2*Δ mutant strain.

Other techniques: Synchronization experiments were performed as described in Lottersberger *et al.* (2003). Flow cytometric DNA analysis was determined on a Becton-Dickinson FACScan. To stain actin cytoskeleton, cells were treated 2 min with 20 units/ml rhodamine-phalloidin (Sigma-Aldrich) in PBS and then washed three times in PBS buffer. Digital images were taken with a CCD camera and software (CoolSNAP; Photometrics). For Western blot analysis, native protein extracts were prepared in 0.1% SDS, 1% Triton, 50 mm Tris pH 7.5, 1 mm sodium deoxycholate, 120 mm β-glycerophosphate, 1.72 mm sodium orthovanadate, 10 mm DTT, 1 mm AEBSF, 15 mm paranitrophenylphosphate, and a protease inhibitor cocktail (Boehringer Mannheim). To detect phosphorylated Mpk1 and Mpk1, polyclonal anti-phospho p42/p44 (Cell Signaling) and anti-Mpk1 (Santa Cruz Biotechnology) antibodies were used, respectively, after 1:1000 dilution in BSA–TBS. Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer.

RESULTS

***MID2*, *WSC2*, *PKC1*, and *GIC1* act as high-dosage suppressors of *bmh* mutants' temperature sensitivity:** We previously generated *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* yeast temperature-sensitive mutants (Lottersberger *et al.* 2003 and Figure 1). In each of our mutant strains, the *bmh1* mutant allele was the sole 14-3-3 source, because all strains carried a *BMH2* deletion, which did not itself cause any of the phenotypes

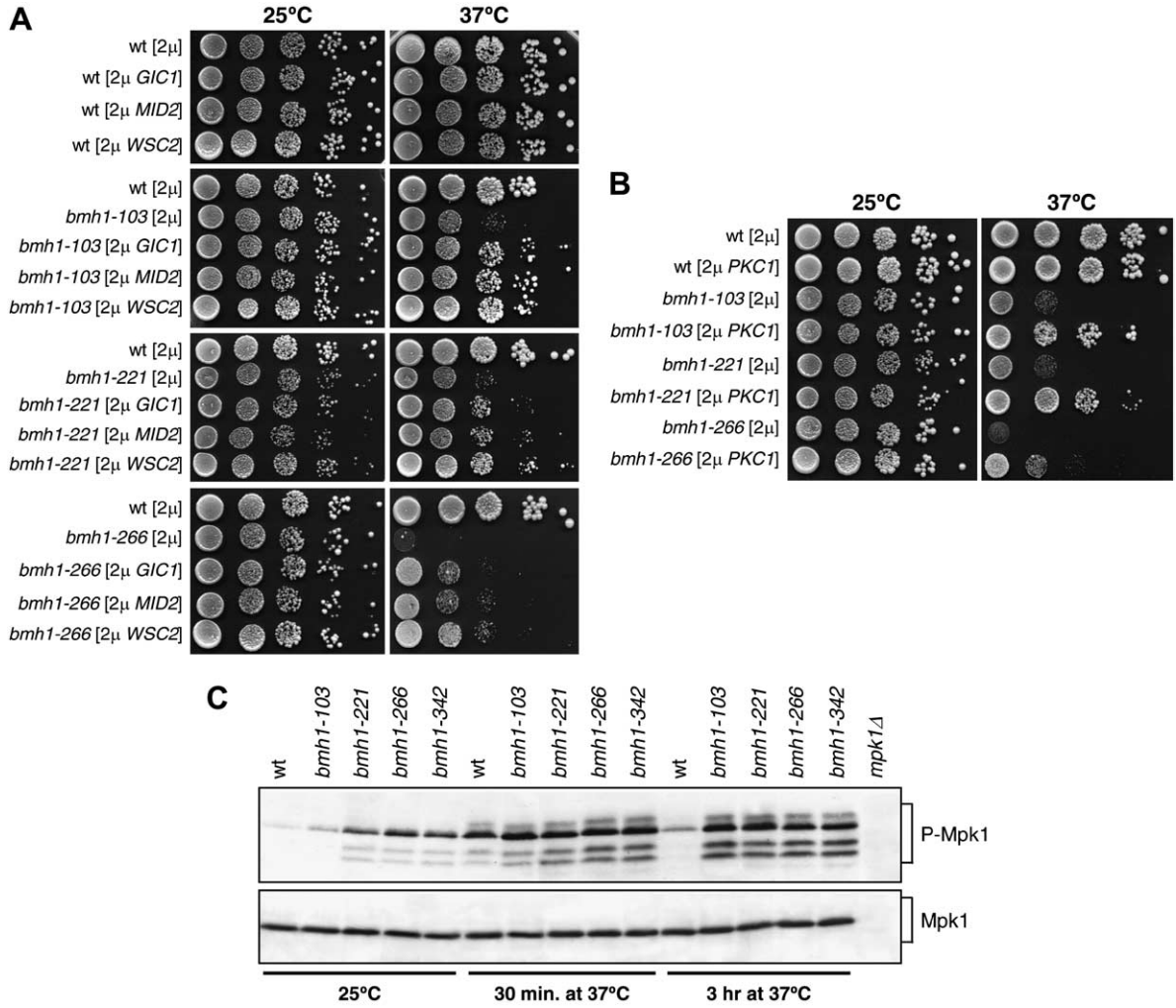


FIGURE 1.—High-dosage suppressors of the *bmh* temperature-sensitive growth defects. (A and B) Exponentially growing cell cultures (selective media at 25°) of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), and *bmh1-266 bmh2*Δ (YLL1120) strains transformed with 2μ plasmids, either empty or carrying the *GIC1*, *MID2*, *WSC2* (A), or *PKC1* (B) genes, were spotted on YEPD plates and incubated at 25° or at 37° for 3 days. (C) Cell cultures of wild type (K699), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ (YLL1092), exponentially growing in YEPD at 25°, were shifted to 37°. Aliquots were withdrawn at time zero (25°) and 30 min or 3 hr after shift at 37° to prepare protein extracts, which were subjected to Western blot analysis with anti-phospho-p44/p42 antibodies (Cell Signaling) to detect Mpk1 phosphorylation (top, P-Mpk1). The two faster migrating bands were likely P-Mpk1 degradation products, and they were not detected by polyclonal antibodies raised against a C-terminal Mpk1 peptide (Santa Cruz Biotechnology), which were used to measure total Mpk1 levels in the same samples (bottom, Mpk1). Specificity of the antibodies was checked by using protein extract prepared from an *mpk1*Δ (YLL1906) strain incubated 30 min at 37°.

described during this study (data not shown). Importantly, overproduction of the Tpk1 catalytic subunit of PKA was unable to suppress the growth defects of these *bmh* mutants at 37° (data not shown), in contrast with its ability to suppress cell lethality caused by Bmh depletion at 25° (GELPERIN *et al.* 1995). Therefore, our *bmh* mutants are not solely impaired in the activation of the Ras/cAMP-dependent PKA pathway.

To identify cellular partners for 14-3-3 proteins, we searched for high-dosage suppressors of the temperature sensitivity of the *bmh1-266* mutant. To this purpose, *bmh1-266* cells were transformed with an *S. cerevisiae* genomic library constructed in the YEp24 2μ vector (CARLSON and BOTSTEIN 1982), and 40,000 Ura^+ transformants were screened for the ability to form colonies on YEPD plates at 37° (see MATERIALS AND METHODS). In addition to 190 *BMH2*- and 20 *BMH1*-bearing plasmids, the screen allowed the recovery of 10 plasmids carrying different ORFs. Subcloning of the several ORFs carried by 3 of these plasmids in the YEplac195 2μ vector revealed that high copy number of the *GIC1*, *WSC2*, or *MID2* genes could partially suppress the temperature sensitivity of *bmh1-266*, *bmh1-103*, and *bmh1-221* cells (Figure 1A). Unfortunately, we were unable to assess this suppressing ability in *bmh1-342* cells due to their high frequency of 2μ plasmid loss.

While the *GIC1* and *GIC2* genes encode two homologous proteins required for actin polarization and bud formation (Brown *et al.* 1997; Chen *et al.* 1997), *WSC2* and *MID2* gene products are transmembrane cell surface sensors. They have been proposed to perform partially overlapping functions in cell wall remodeling during vegetative growth and under stress conditions (Verna *et al.* 1997; Marcoux *et al.* 1998; Ketela *et al.* 1999; Rajavel *et al.* 1999; Philip and Levin 2001) and to detect and signal the cell wall status to Pkc1. The latter is involved in a multiplicity of pathways, including those related to bud emergence, cell wall integrity, and organization of the actin cytoskeleton, in response to heat shock, pheromone, low osmolarity, nutrient starvation, and cell cycle progression (Heinisch *et al.* 1999). We therefore examined whether an excess of Pkc1 could also suppress the temperature sensitivity of our *bmh* mutants. Indeed, high copy number of *PKC1* improved the ability of *bmh1-103*, *bmh1-221*, and *bmh1-266* cells to form colonies at 37° (Figure 1B), indicating that hyperactivation of a Pkc1-dependent cascade may compensate for defects in 14-3-3 proteins.

Pkc1 is believed to possess multiple functions (Lee and Levin 1992; Verna *et al.* 1997; Delley and Hall 1999; Ketela *et al.* 1999; Andrews and Stark 2000; Zanelli and Valentini 2005), only one of which is to regulate the activity of the MAPK cascade that ultimately regulates cell wall integrity, bud emergence, response to hypotonic shock, and actin reorganization (reviewed in Levin and Errede 1995). Because high levels of Wsc2, Mid2, or Pkc1 (predicted to result in increased signaling through Pkc1) suppressed the temperature-sensitive growth defects of our *bmh* mutants, we asked whether the latter were defective in activating the Pkc1-dependent MAPK cascade. To this end, we monitored Mpk1 phosphorylation, which is an established marker for activation of the Pkc1–MAPK pathway (Lee *et al.* 1993; Zarzov *et al.* 1996; de Nobel *et al.* 2000). As shown in Figure 1C, the amount of phosphorylated Mpk1 in *bmh* mutants was higher than in wild type already at the permissive temperature. Moreover, heat shock, which is known to induce a rapid transient depolarization of the actin cytoskeleton and cell wall weakening (Delley and Hall 1999), increased the amount of Mpk1 phosphorylated forms in both wild-type and *bmh* mutants after a shift to 37° for 30 min (Figure 1C). However, phosphorylated Mpk1 level significantly decreased in wild-type cells within 3 hr at 37°, due to adaptation to the high temperature, whereas it remained high in *bmh* cells under the same conditions (Figure 1C). Thus, the growth defects of our *bmh* mutants are unlikely due to faulty MAPK signaling, as the latter appears to be hyperactivated instead in these mutants. Rather, these mutants could be deficient in some Pkc1-regulated pathway that parallels the one involving MAP kinases. In this view, the MAPK cascade could be hyperactivated in *bmh* mutants as a compensatory mechanism to maintain cell viability in the absence of 14-3-3 function. Accordingly, we found that *MPK1* deletion was lethal for the *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* mutants (data not shown). Therefore, an excess of Wsc2, Mid2, or Pkc1 may suppress temperature sensitivity of *bmh* mutants by acting through a Pkc1-dependent MAPK-independent pathway.

Temperature-sensitive *bmh* mutants are defective in the G_1/S transition and actin polarization: Because enhanced Pkc1 signaling contributes to cell viability in the absence of 14-3-3 function, we asked whether *bmh* mutants were impaired in Pkc1-regulated cellular processes, such as bud formation, actin reorganization, cell wall remodeling, and cell cycle progression (Heinisch *et al.* 1999). To investigate whether defects in 14-3-3 functions may affect bud formation at the G_1/S transition, exponentially growing cultures of wild-type, *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* cells were arrested in G_1 with α-factor at 25° and then released into fresh medium at 37°. As shown in Figure 2A, most of *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* cells were still largely unbudded after 1 hr at 37°, when bud emergence had already occurred in 90% of similarly treated wild-type cells. After 3 hr at 37°, most *bmh1-221* and *bmh1-342* mutant cells were still unbudded, while ~50% of *bmh1-103* and *bmh1-266* cells managed to bud (Figure 2, A and B). However, their buds appeared mostly misshapen and, upon further incubation at 37°, continued to elongate (Figure 2, A and B). Moreover, some elongated budded cells appeared also in *bmh1-221* and *bmh1-342* mutants at later time points. Thus, *bmh* mutants might be impaired in the switch between apical to isotropic growth.

Delayed bud formation in *bmh* mutants was parallel with defects in DNA synthesis initiation. In fact, all *bmh* mutants severely delayed initiation of DNA replication, although to different extents after a shift to 37° (Figure 2C). In fact, the onset of DNA replication took place in *bmh1-221* and *bmh1-342* cells only ~150 min after release at 37° from the G_1 block, while wild-type cells initiated DNA replication after 45–60 min under the same conditions (Figure 2C). In addition, a major fraction of cells in both mutants were unable to replicate DNA by 240 min (Figure 2C). The *bmh1-103* and *bmh1-266* cells started DNA replication ~120 and 75 min after release at 37°, respectively, and again only a fraction of these mutant cells managed to complete DNA replication by 240 min at 37° (Figure 2C). As shown in Figure 2D, initiation of DNA replication upon release at 25° of the same G_1-arrested cell cultures was delayed by 15–30 min in *bmh* mutant cell cultures compared to wild type. Altogether, these data indicate that 14-3-3 proteins are required for a timely G_1/S transition.

Both bud emergence and its subsequent surface growth require the polarization of the actin cytoskeleton, such that cortical patches and actin cables converge at the bud site (reviewed in Pruyne and Bretscher

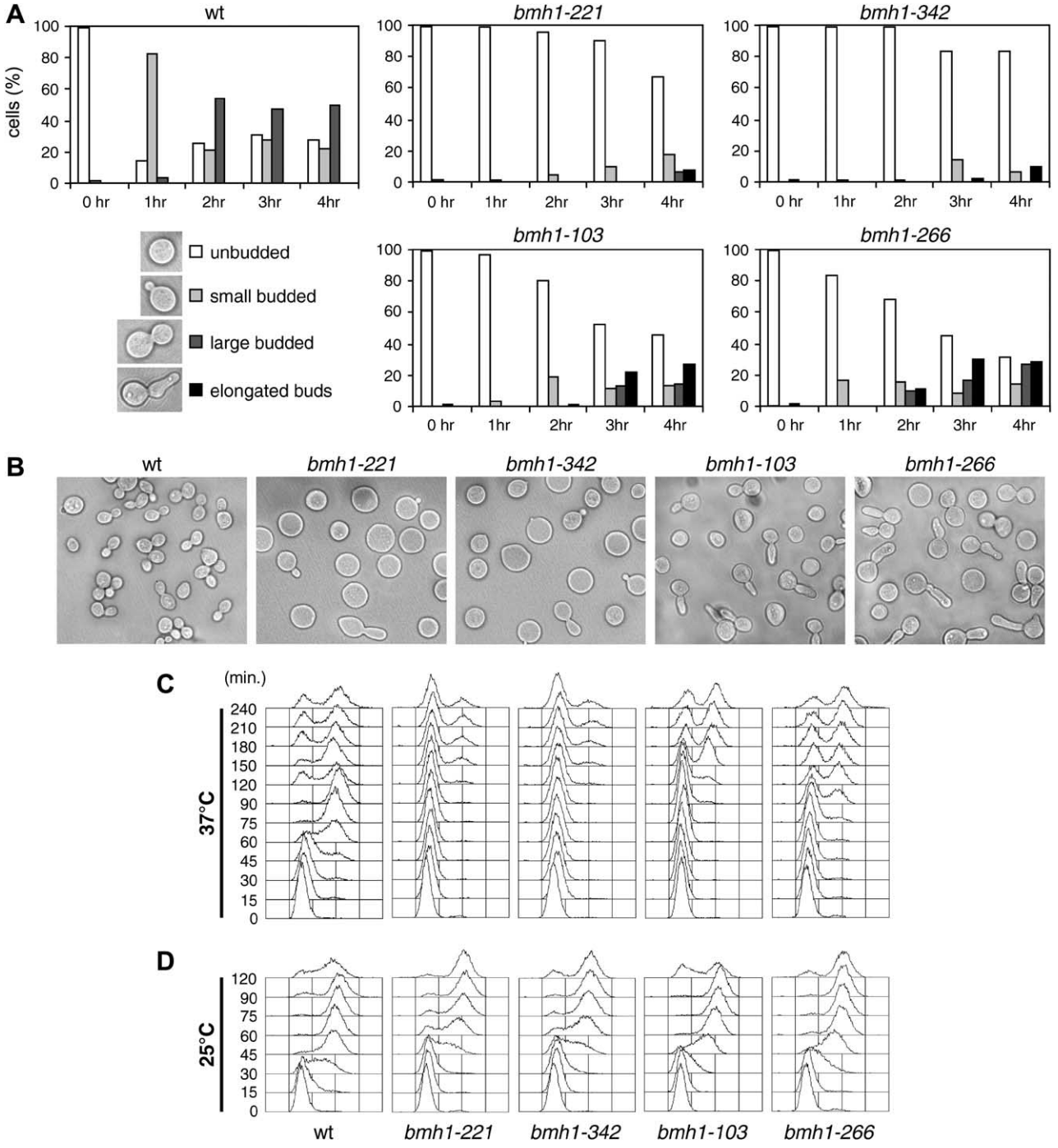


Figure 2.—Temperature-sensitive *bmh* mutants are defective in bud emergence and initiation of DNA replication. Cell cultures of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ (YLL1092) strains, exponentially growing at 25° in YEPD, were arrested in G_1 with α-factor for 2 hr and released at time zero in YEPD at 25° or 37°. (A) A total of 200 cells for each strain were analyzed to determine the frequency of cells with no, small, large, or elongated buds at 25° (0 hr) and at the indicated time points after shift at 37°. (B) Photographs were taken 3 hr after shift at 37°. (C and D) To determine DNA contents by fluorescence-activated cell sorting (FACS) analysis, samples were withdrawn at the indicated times after release in YEPD at 37° (C) or at 25° (D).

2000). Since 14-3-3 proteins have been previously linked to actin cytoskeleton organization (Gelperin *et al.* 1995; Roth *et al.* 1999), impaired bud formation in the above *bmh* mutants might be related to defects in this process. To address this issue, we analyzed actin polarization upon Alexa-Fluor 546 phalloidin staining of wild-type and *bmh* mutant cells arrested in G_1 by α-factor and then released at 37° for 1 hr. As shown in Figure 3, actin cortical patches, which normally clustered at the bud tips of wild-type cells, were completely missing in *bmh1-221*

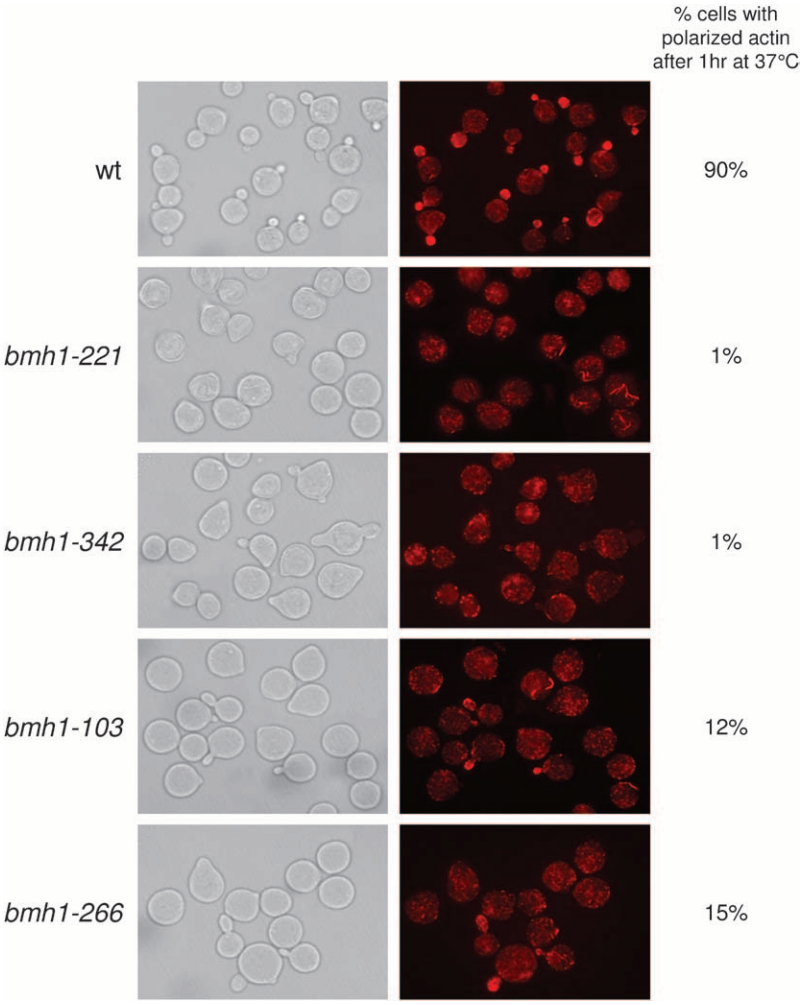


FIGURE 3.—Actin organization in the temperature-sensitive *bmh* mutants. Cell cultures of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ (YLL1092) strains, exponentially growing at 25° in YEPD, were synchronized in G_1 with α-factor and released at time zero in YEPD at 37°. Cells were fixed 1 hr after the release at 37°, stained with fluorochrome-conjugated phalloidin, and scored for the presence of cells with polarized actin by fluorescence microscopy. Differential interference contrast (left) and epifluorescence (right) images are shown as examples.

and *bmh1-342* cells and appeared only in a small fraction of *bmh1-103* and *bmh1-266* cells. Therefore, organization of the actin cytoskeleton at the future bud emergence sites is perturbed in *bmh* mutants, thus affecting bud formation at the G_1/S transition.

We then combined the different *bmh1* alleles, together with the *BMH2* deletion, with the temperature-sensitive *cdc42-1* or *cdc24-1* alleles, altering the essential Cdc42 GTPase and its guanine-nucleotide-exchange factor (GEF) Cdc24 (ADAMS *et al.* 1990; JOHNSON and PRINGLE 1990; VAN AELST and D'SOUZA-SCHOREY 1997), which are both required to establish actin cytoskeleton polarity (reviewed in PRUYNE and BRETSCHER 2000). As shown in Figure 4, the ability of all *bmh1 bmh2*Δ *cdc24-1* and *bmh1 bmh2*Δ *cdc42-1* triple mutants to form colonies at 32° was severely impaired compared to that of the parental mutants. This synthetic effect between *bmh* and *cdc24* or *cdc42* mutant alleles further supports a role for budding yeast 14-3-3 proteins in actin polarization and bud formation.

Bmh defects cause sensitivity to cell wall stress and their effects on the G_1/S transition can be relieved by osmotic support: Pkc1 controls cell wall metabolism by regulating both β-glucan synthesis at the site of wall remodeling and expression of cell wall biosynthesis genes necessary for maintaining cellular integrity during bud formation and in response to heat shock, pheromone, and nutrient starvation (reviewed in LEVIN 2005). We therefore asked whether defects in 14-3-3 functions might result in impaired cell wall integrity. We analyzed the ability of *bmh* mutants to grow at permissive temperature in the presence of compounds such as the chitin antagonist calcofluor white and SDS. In fact, both compounds are powerful tools for revealing yeast cell wall defects (RAM *et al.* 1994). As shown in Figure 5A, *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* cells were unable to grow on YEPD plates supplemented with 0.01% SDS, which did not affect wild-type cell growth. Moreover, growth of all *bmh* mutants on YEPD was compromised, although to different degrees, by addition of 0.01 mg/ml calcofluor white (Figure 5B). Finally, microscopic examination of the *bmh* mutant cell cultures revealed accumulation of cell debris at 37° (data not shown), suggesting that cell lysis frequently occurred. Thus, 14-3-3 proteins appear to be required for a stable cell wall structure.

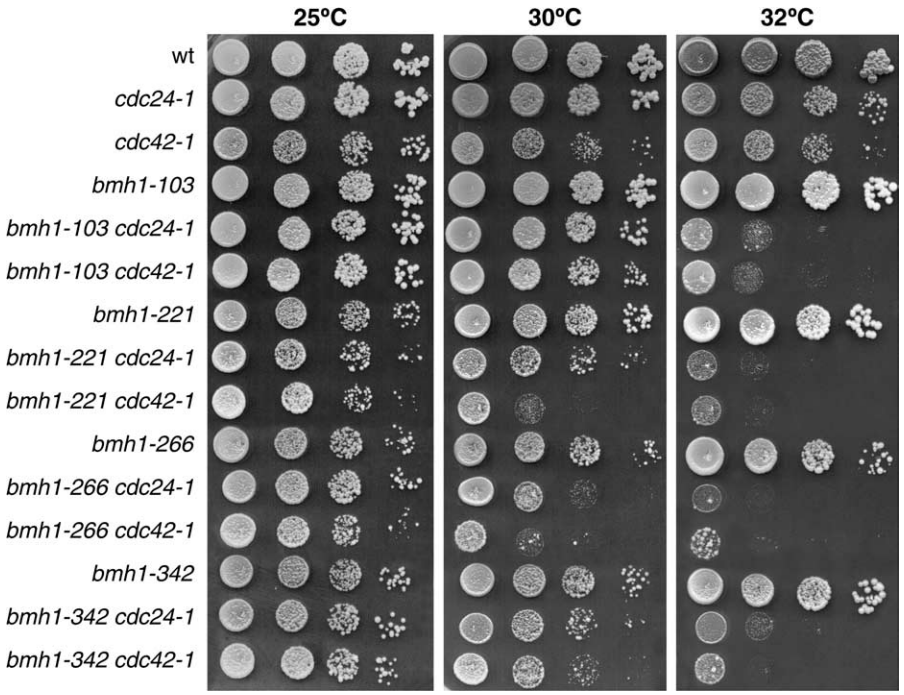


FIGURE 4.—Synthetic effects between *bmh* and polarization mutant alleles. The following strains were used: wild type (W303), *cdc24-1*, *cdc42-1*, *bmh1-103 bmh2*Δ (YLL1082), *bmh1-103 cdc24-1 bmh2*Δ (DMP4436/3C), *bmh1-103 cdc42-1 bmh2*Δ (DMP4430/7A), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-221 cdc24-1 bmh2*Δ (DMP4439/6B), *bmh1-221 cdc42-1 bmh2*Δ (DMP4433/10B), *bmh1-266 bmh2*Δ (YLL1120), *bmh1-266 cdc24-1 bmh2*Δ (DMP4440/5C), *bmh1-266 cdc42-1 bmh2*Δ (DMP4434/7B), *bmh1-342 bmh2*Δ (YLL1092), *bmh1-342 cdc24-1 bmh2*Δ (DMP4441/5A), and *bmh1-342 cdc42-1 bmh2*Δ (DMP4435/4B). Serial dilutions of cell cultures, exponentially growing in YEPD at 25°, were spotted on YEPD plates and incubated at the indicated temperatures for 3 days.

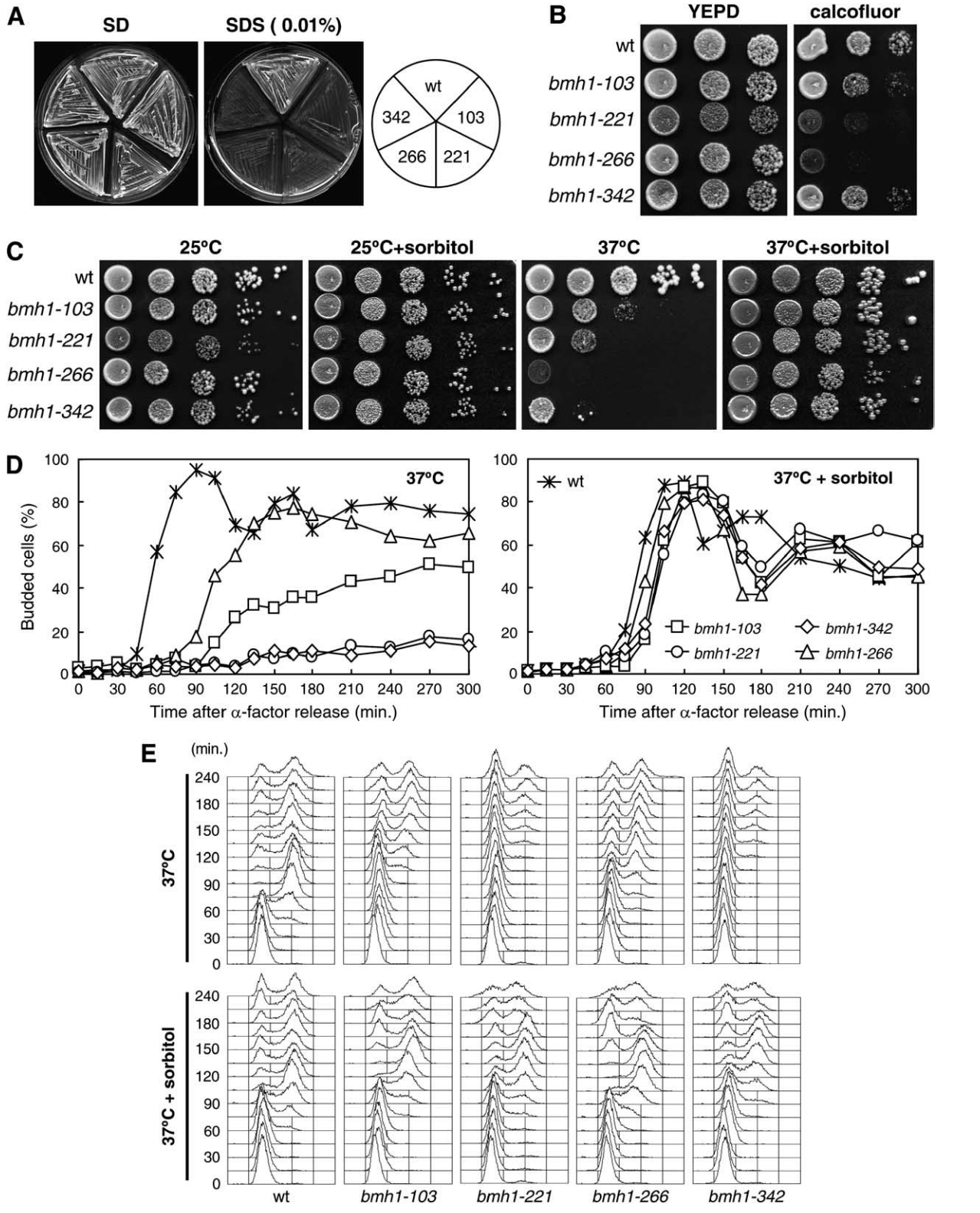
We therefore examined whether osmotic stabilization of the medium might relieve the temperature sensitivity and the G_1/S transition defects of our *bmh* mutants. As shown in Figure 5C, addition of the osmotic stabilizer sorbitol restored the ability of *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* cells to grow on YEPD plates at 37°. Moreover, the presence of sorbitol in the medium largely rescued the defects in bud emergence (Figure 5D) and initiation of DNA replication (Figure 5E) displayed by *bmh* mutants upon G_1 release at 37°. Thus, slow growth and delayed G_1/S transition that are caused by defective 14-3-3 proteins are osmoremediable, suggesting a primary defect of *bmh* mutants in cell wall biogenesis.

Wsc2, Mid2, or Pkc1 high dosage can partially suppress the G_1/S transition defects of *bmh* mutants: Because enhanced Pkc1-dependent signaling by high copy number *WSC2*, *MID2*, or *PKC1* suppressed the temperature sensitivity of *bmh* mutants, we asked whether it also suppressed their G_1/S transition defects. As shown in Figure 6A, *bmh1-103*, *bmh1-221*, and *bmh1-266* cells carrying *WSC2*, *MID2*, or *PKC1* on a 2μ plasmid and released from α-factor at 37° underwent budding more efficiently than the same mutant cells carrying the empty vector. Moreover, an excess of Wsc2, Mid2, or Pkc1 attenuated the abnormal bud morphology of *bmh1-103* and *bmh1-266* cells after 3 hr at 37° (Figure 6A). Similarly, bud emergence took place more efficiently in *bmh1-221*, *bmh1-103*, and *bmh1-266* cells containing an excess of Gic1, although this, as expected, caused bud elongation even in wild-type cells due to sustained polarized growth (Figure 6A) (BROWN *et al.* 1997; CHEN *et al.*, 1997).

Suppression of *bmh* defects in DNA replication initiation was also apparent upon *WSC2*, *MID2*, or *PKC1* increased dosage. In fact, *bmh1-103*, *bmh1-221*, and *bmh1-266* cells carrying high copy number *WSC2*-, *MID2*-, or *PKC1*-bearing plasmids initiated DNA replication at 37° earlier and more efficiently than the same mutants with the empty vector (Figure 6B). Thus, an excess of Wsc2, Mid2, or Pkc1 can partially suppress the G_1/S transition defects of *bmh* mutants.

Low G_1 cyclin-Cdk1 levels may account for the G_1/S transition defects of *bmh* mutants: During the G_1/S transition, Pkc1 acts in concert with the SBF transcription factor to control the actin cytoskeleton, cell cycle progression, and transcription of cell wall biosynthesis genes (reviewed in LEVIN 2005). SBF is composed of the Swi6 and Swi4 subunits and is responsible for transcriptional activation of the *CLN1* and *CLN2* cyclin genes, whose products associate with the cyclin-dependent kinase 1 (Cdk1) to promote bud morphogenesis and DNA replication (reviewed in LEVIN *et al.* 1995; NASMYTH 1996).

FIGURE 5.—Temperature-sensitive *bmh* mutants are sensitive to cell wall stress. (A) Serial dilutions of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ (YLL1092) cell cultures, exponentially growing in YEPD at 25°, were streaked on SD plates with or without SDS (0.01%). (B) The same cultures in A were spotted on YEPD plates with or without Calcofluor (0.01 mg/ml). Plates were incubated at 25° for 4 days. (C) Serial dilution of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ



(YLL1092) cell cultures, exponentially growing in YEPD at 25°, were spotted on YEPD plates in the absence or presence of 1 M sorbitol and incubated at the indicated temperatures for 3 days. (D and E) Cell cultures of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-266 bmh2*Δ (YLL1120), and *bmh1-342 bmh2*Δ (YLL1092) strains, exponentially growing at 25° in YEPD, were arrested in G_1 with α-factor for 2 hr and released at time zero in YEPD at 37° in the absence or presence of 1 M sorbitol. Samples were withdrawn at the indicated times after α-factor release to analyze the kinetics of bud emergence (D) and DNA contents by FACS analysis (E).

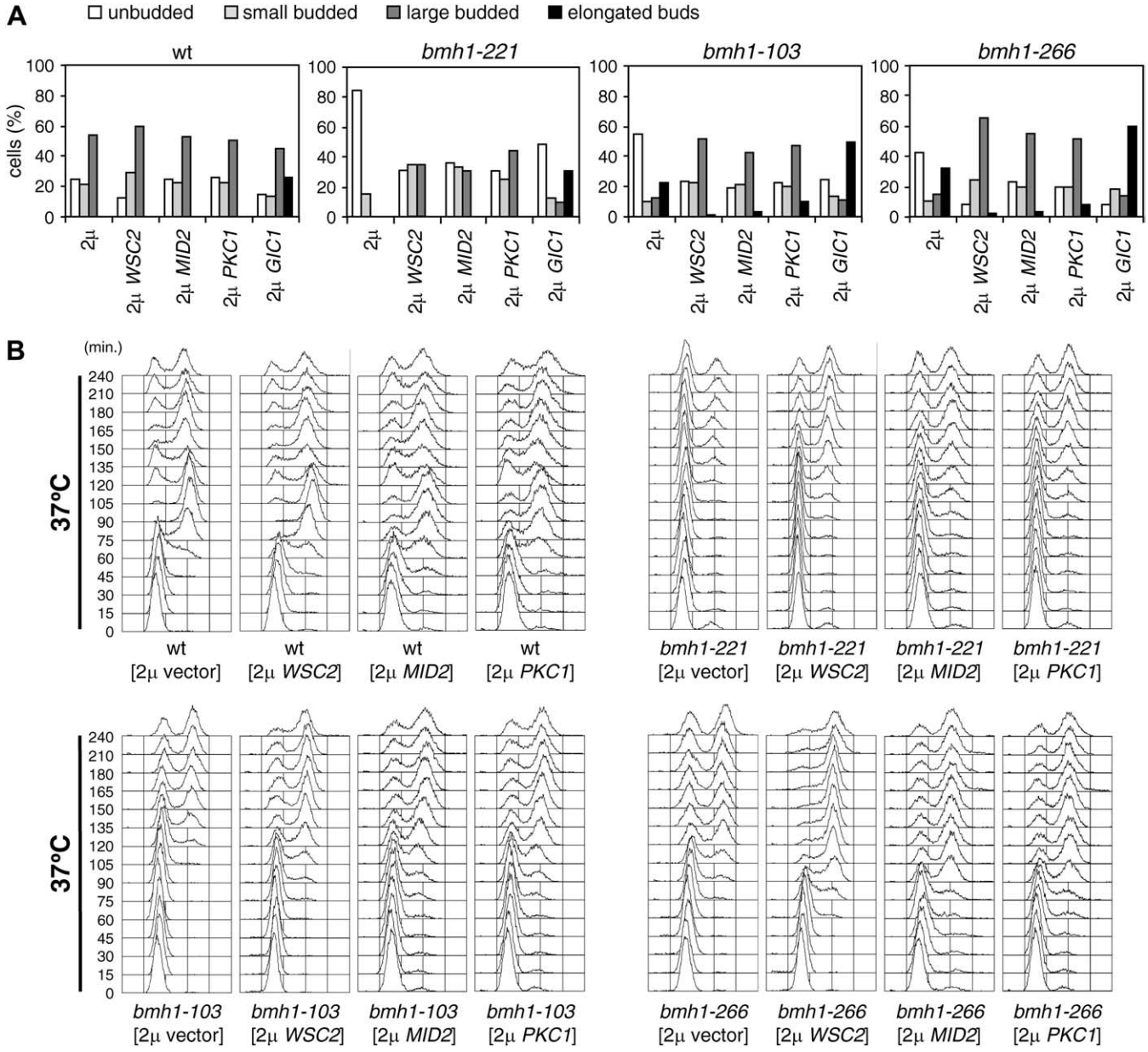


FIGURE 6.—*WSC2*, *MID2*, and *PKC1* overexpression can suppress the G_1/S transition delay of *bmh* mutants. Exponentially growing (selective media at 25°) cell cultures of wild-type (W303), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-221 bmh2*Δ (YLL1081), and *bmh1-266 bmh2*Δ (YLL1120) strains transformed with 2μ plasmids, either empty or carrying the *WSC2*, *MID2*, *PKC1*, or *GIC1* genes, were arrested in G_1 with α-factor and released at time zero in YEPD at 25° or 37°. (A) A total of 200 cells for each strain were analyzed to determine the frequency of cells with no, small, large, or elongated buds after 3 hr at 37°. (B) Samples were withdrawn at the indicated times after α-factor release to analyze DNA contents by FACS analysis.

Since Pkc1 hyperactivation was shown to partially compensate for the lack of SBF activity (GRAY *et al.* 1997; IGUAL *et al.* 1996), we asked whether the G_1/S transition defects of *bmh* mutants might be related to impaired formation of G_1 cyclin/Cdk1 complexes. We therefore measured the levels of *CLN2* mRNA in the *bmh1-221* and *bmh1-342* mutants, which showed the most severe G_1/S transition defects at 37° when compared to the other *bmh* mutants (Figure 2). Exponentially growing cultures of wild-type, *bmh1-221*, and *bmh1-342* cells were arrested in G_1 with α-factor and released into the cell cycle at 37°. Total RNA was prepared at different time points after release and subjected to Northern blot analysis with a *CLN2* probe. As shown in Figure 7A, *CLN2* mRNA was detectable in wild-type cells starting 30–45 min after release, just before bud emergence (data not shown) and initiation of DNA replication. Conversely, its amount was dramatically reduced in both *bmh1-221* and *bmh1-342* mutant cells that remained arrested with 1C DNA contents for at least 180 min after release at 37° (Figure 7A). If the G_1/S transition defects of our *bmh* mutants were due to low amounts of G_1 cyclin/Cdk1

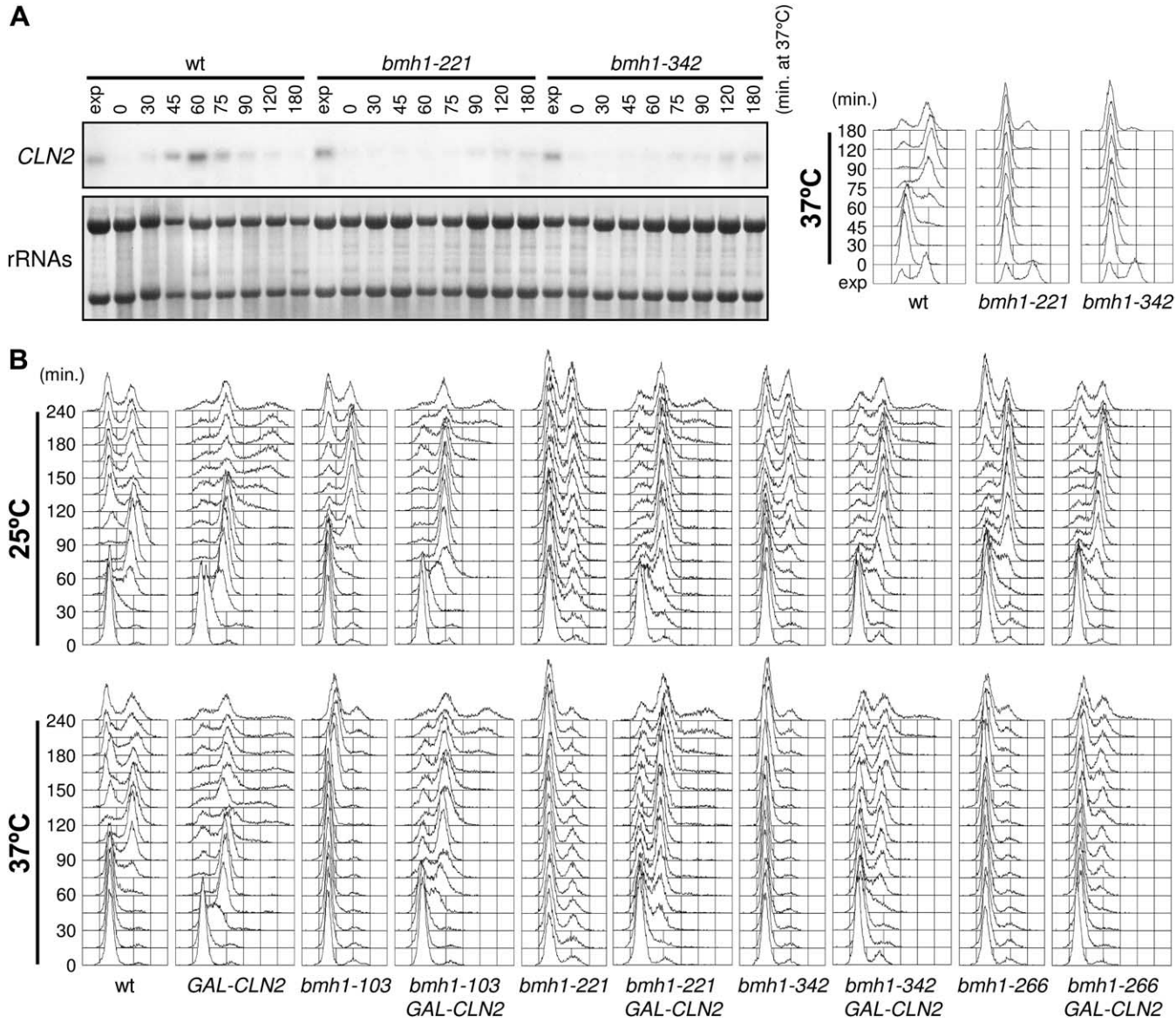


Figure 7.—*CLN2* mRNA levels and *CLN2* ectopic expression in *bmh* mutants. (A) Cell cultures of wild-type (W303), *bmh1-221 bmh2*Δ (YLL1081), and *bmh1-342 bmh2*Δ (YLL1092) strains, exponentially growing in YEPD at 25°, were synchronized in G_1 with α-factor and released at time zero into YEPD at 37°. Samples were taken at the indicated times after the release into the cell cycle to analyze *CLN2* mRNA by Northern analysis (left) and to determine DNA contents by FACS analysis (right). Loading control of the Northern blot is a methylene blue-stained filter of ribosomal RNAs (rRNAs). (B) Cell cultures of wild-type (W303), *GAL-CLN2* (DMP4357/1B), *bmh1-103 bmh2*Δ (YLL1082), *bmh1-103 bmh2*Δ *GAL-CLN2* (DMP4370/2D), *bmh1-221 bmh2*Δ (YLL1081), *bmh1-221 bmh2*Δ *GAL-CLN2* (DMP4372/3B), *bmh1-266 bmh2*Δ (YLL1120), *bmh1-266 bmh2*Δ *GAL-CLN2* (DMP4373/7C), *bmh1-342 bmh2*Δ (YLL1092), and *bmh1-342 bmh2*Δ *GAL-CLN2* (DMP4465/3B) strains, exponentially growing in YEP+raffinose at 25°, were synchronized in G_1 with α-factor for 2 hr. Galactose was added 30 min before release. Synchronized cells were then released at time zero into YEP+raf+gal at 25° (top) or at 37° (bottom). Samples were taken at the indicated times after release to determine DNA contents by FACS analysis.

complexes caused by the reduced *CLN1* and *CLN2* mRNA levels, *CLN2* expression from an ectopic promoter might suppress the G_1/S transition defects of our *bmh* mutants. To test this hypothesis, cultures of wild-type, *bmh1-103*, *bmh1-221*, *bmh1-266*, and *bmh1-342* strains, carrying or lacking a galactose-inducible *GAL1-CLN2* construct, were grown in YEP+raffinose at 25°, arrested in G_1 with α-factor, and then released at 25° or 37° in galactose-containing medium to induce *CLN2* expression. *GAL1-CLN2* induction significantly rescued the G_1/S defects of most *bmh* mutants. In fact, both bud emergence (data not shown) and initiation of DNA replication (Figure 7B) were advanced upon galactose induction in all *bmh GAL1-CLN2* strains compared to the isogenic *bmh* strains, at both 25° and 37°. In particular, S phase entry took place in *GAL-CLN2*, *bmh1-103 GAL-CLN2*, *bmh1-221 GAL-CLN2*, and *bmh1-342 GAL-CLN2* strains at 30, 45, 30, and 60 min, respectively, after

release at 37° in galactose-containing medium. Similarly treated *bmh1-103*, *bmh1-221*, and *bmh1-342* cells neither budded (data not shown) nor initiated DNA replication up to 4 hr after release (Figure 7B). Conversely, ectopic *CLN2* expression had only a marginal effect on *bmh1-266* cells, allowing only 30% of them to initiate DNA replication by 4 hr at 37° (Figure 7B). This suggests that functions other than activation of Cln1, 2/Cdk1 might be affected in this mutant. It is worth noting that expression of *CLN2* from the *GAL1* promoter caused cytokinesis defects at late time points in most strains, leading to accumulation of cells with elongated buds (data not shown) and more than 2C DNA contents (Figure 7B), as previously reported (Lew and Reed 1993). Altogether, these data indicate that reduced amounts of G_1 cyclin/Cdk1 complexes may partially account for the G_1/S transition defects of our *bmh* mutants.

DISCUSSION

To understand the essential function(s) of *S. cerevisiae* 14-3-3 proteins, we searched for high dosage suppressors of the temperature sensitivity of *bmh* mutants, carrying *bmh1* mutant alleles as the sole 14-3-3 sources (Lottersberger *et al.* 2003). We found that the growth defects of *bmh1-103*, *bmh1-221*, and *bmh1-266* at 37° can be rescued by overproducing Pkc1 or its transmembrane cell surface sensors Wsc2 and Mid2. The latter have been proposed to perform partially overlapping functions in cell wall remodeling during vegetative growth and under stress conditions by detecting and transmitting cell wall status to Pkc1 (Verna *et al.* 1997; Ketela *et al.* 1999; Rajavel *et al.* 1999; Philip and Levin 2001). Pkc1 is believed to possess multiple functions (Lee and Levin 1992; Verna *et al.* 1997; Delley and Hall 1999; Ketela *et al.* 1999; Andrews and Stark 2000; Zanelli and Valentini 2005), one of which is to regulate the MAPK cascade involved in cell wall construction and polarized growth (reviewed in Levin and Errede 1995). On the basis of Mpk1 phosphorylation, the Pkc1-dependent MAPK cascade appears to be hyperactivated at both 25° and 37° in our *bmh1* mutants, suggesting that defects in 14-3-3 proteins affect a pathway that is regulated by Pkc1 but does not involve Mpk1. Thus, Wsc2, Mid2, or Pkc1 may act as high-dosage suppressors by stimulating the former pathway, whereas the hyperactivation of the MAPK cascade in our mutants could be the result of a compensatory mechanism that contributes to their cell viability at the permissive temperature. Accordingly, deletion of *MPK1* was lethal for our *bmh* mutants, indicating that 14-3-3 proteins and Mpk1 act in different branches of the Pkc1 pathway to sustain cell viability.

Pkc1, together with its upstream regulators Wsc1-3 and Mid2, controls actin cytoskeleton reorganization, cell cycle progression, and transcription of cell wall biosynthesis genes involved in synthesis and assembly of cell wall components at the bud (reviewed in Levin 2005). Since enhanced Pkc1-dependent signaling can partially suppress the temperature sensitivity of *bmh* mutants, some of the above Pkc1-regulated processes might be impaired in these mutants. Indeed, we found that all our temperature-sensitive *bmh1* alleles cause defects in G_1/S transition, actin polarization at the prebud site, and cell wall integrity. In fact, a shift to the restrictive temperature severely impairs bud formation and initiation of DNA replication in *bmh1-221* and *bmh1-342* mutants and significantly slows the same processes in *bmh1-103* and *bmh1-266* cells. When the entry into S phase and bud emergence take place in some of the latter mutant cells, buds are elongated, suggesting a defective apical–isotropic switch in bud growth. Consistent with a function for 14-3-3 proteins in bud formation and actin polarization, *bmh* mutant alleles also cause synthetic effects at semipermissive temperature when combined with the *cdc42-1* and *cdc24-1* temperature-sensitive alleles. These alleles alter the Rho-family GTPase Cdc42 and its GEF, respectively, which are essential for polarizing the actin cytoskeleton (reviewed in Pruyne and Bretscher 2000). Moreover, high levels of the Cdc42 effector Gic1, which binds to the activated GTP-bound form of Cdc42 and is required for cytoskeletal polarization during bud emergence (Chen *et al.* 1997; Brown *et al.* 1997), can partially suppress the temperature sensitivity of *bmh1-103*, *bmh1-221*, and *bmh1-266* mutants. Finally, our *bmh* mutants undergo cell lysis at the restrictive temperature and are hypersensitive to calcofluor and SDS at the permissive temperature, suggesting that they are impaired in cell wall integrity. In agreement with a 14-3-3 role in cell wall biogenesis, both the growth defects and the G_1/S transition delay at 37° of our *bmh* mutants can be rescued by the addition of the osmostabilizer sorbitol.

Both initiation of DNA replication and bud morphogenesis require activation of G_1 cyclin/Cdk1 complexes (reviewed in Nasmyth 1996). In particular, when cells reach a critical size, Cln3-Cdk1 activates the SBF transcription factor that induces transcription of the *CLN1* and *CLN2* genes (Nasmyth and Dirick 1991; Ogas *et al.* 1991; Cross *et al.* 1994; Dirick *et al.* 1995). The Pkc1-dependent cascade acts in concert with SBF to control the actin cytoskeleton and transcription of cell wall biosynthesis genes involved in maintaining cellular integrity during bud formation (Levin and Bartlett-Heubusch 1992; Lew and Reed 1993; Mazzoni *et al.* 1993; Igual *et al.* 1996; Marini *et al.* 1996; Zarzov *et al.* 1996; Gray *et al.* 1997; Madden *et al.* 1997; Delley and Hall 1999). Accordingly, *swi4*Δ and *swi6*Δ mutants are sensitive to cell wall stresses and the growth defects of *swi4*Δ cells can be partially relieved by osmotic stabilization, supporting a role for SBF in cell wall biogenesis (Igual *et al.* 1996; Gray *et al.* 1997). Moreover, *swi4* and *pkc1* mutations are synthetically lethal (Madden *et al.* 1997), whereas the temperature-sensitive growth of

*swi4*Δ cells can be suppressed by overproduction of Pkc1 or Wsc1, the latter belonging to the Wsc1-3 family of transmembrane proteins required for heat stress activation of the Pkc1-MAPK cascade (Igual *et al.* 1996; Gray *et al.* 1997). Finally, Pkc1 seems to play redundant functions with G_1 cyclins since deletion of *PKC1* causes cell death in a *cln1*Δ *cln2*Δ double mutant (Gray *et al.* 1997).

The partially redundant function of Pkc1 and SBF-dependent pathways and the similarities in the behavior of *bmh* and *swi4* or *swi6* mutants raise the possibility that the phenotypes of *bmh* mutants might arise from defective SBF activity. In agreement with this hypothesis, Cln1, 2/Cdk1 complexes appear to be limiting for execution of the G_1/S transition in *bmh* mutants. In fact, the amount of *CLN2* mRNA is dramatically reduced in both *bmh1-103* and *bmh1-342* mutants at 37° compared to wild type. Moreover, expression of *CLN2* from an SBF-independent promoter can partially suppress *bmh* mutant defects in budding and DNA replication. Consistent with the possibility that defective 14-3-3 proteins may impair SBF-dependent accumulation of Cln1, 2/Cdk1 complexes, a mutation in the *SIN4* gene, whose lack of function bypasses the requirement for Swi4 and Swi6 to transcribe the *HO-LacZ* reporter gene (Nasmyth *et al.* 1987; Lycan *et al.* 1994; Li *et al.* 2005), was shown to suppress the temperature sensitivity of a *bmh2 bmh1*Δ mutant (van Heusden and Steensma 2001). Since SBF-dependent induction of Cln1, 2/Cdk1 triggers both entry into S phase by turning on proteolysis of the cyclin B-Cdk1 inhibitor Sic1 and cytoskeleton polarization for bud formation (Lew and Reed 1993; Schwob *et al.* 1994; Tyers 1996), impaired SBF-dependent Cln1, 2/Cdk1 complex formation may account for both budding and DNA replication defects of our *bmh* mutants. It should in fact be noted that, unlike in most of the other laboratory strains, simultaneous deletion of *CLN1* and *CLN2* is lethal in the W303 genetic background that we used for all our experiments (Cvrckova *et al.* 1995). Therefore, low levels of Cln1, 2/Cdk1 complexes in *bmh* mutants may be the cause of their G_1/S transition defects at high temperatures.

In any case, since SBF and Pkc1 play a partially redundant role in allowing bud formation and cell integrity (Igual *et al.* 1996; Gray *et al.* 1997; Madden *et al.* 1997), hyperactivation of the Pkc1-dependent cascades by high levels of Wsc2, Mid2, or Pkc1 might suppress the growth and G_1/S transition defects of *bmh* mutants by partially compensating defects in SBF activity and G_1 cyclin/Cdk1 complex accumulation.

Taken together, these data indicate that *S. cerevisiae* 14-3-3 proteins play essential functions in regulating processes that occur at the G_1/S transition. Since 14-3-3 proteins are highly conserved in evolution, our studies may also help to elucidate their essential functions in higher eukaryotes.

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