Feedback Inhibition on Cell Wall Integrity Signaling by Zds1 Involves Gsk3 Phosphorylation of a cAMP-dependent Protein Kinase Regulatory Subunit*

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We report here that budding yeast cAMP-dependent protein kinase (cAPK) is controlled by heat stress. A rise in temperature from 30 to 37 °C was found to result in both a higher expression and an increased cytoplasmic localization of its regulatory subunit Bcy1. Both of these effects required phosphorylation of serines located in its localization domain. Surprisingly, classic cAPK-controlled processes were found to be independent of Bcy1 phosphorylation, indicating that these modifications do not affect cAPK activity as such. Alternatively, phosphorylation may recruit cAPK to, and thereby control, a specific subset of (perhaps novel) cAPK targets that are presumably localized extranuclearily. Zds1 and Zds2 may play a role in this process, since these were found required to retain hyperphosphorylated Bcy1 in the cytoplasm at 37 °C. Mck1, a homologue of mammalian glycogen synthase kinase 3 and a downstream component of the heat-activated Pkc1-Slt2/Mpk1 cell wall integrity pathway, is partly responsible for hyperphosphorylations of Bcy1. Remarkably, Zds1 appears to act as a negative regulator of cell wall integrity signaling, and this activity is dependent in part on the phosphorylation status of Bcy1. Thus, Mck1 phosphorylation of Bcy1 and Zds1 may constitute an unprecedented negative feedback control on the cell wall integrity-signaling pathway.

In eukaryotes from yeast to humans, cAMP-dependent protein kinases (cAPKs) are ubiquitous signaling proteins. A structural characteristic of cAPKs is that the regulatory and catalytic activities are not covalently linked but are represented by two different subunits. The inactive cAPK holoenzyme consists of a regulatory (R) subunit homodimer with two catalytic subunits associated to it. Binding of the second messenger cAMP to the R-dimer leads to dissociation of the holoenzyme, and the released catalytic subunits are then able to phosphorlylate protein substrates at serine or threonine residues comprising a defined consensus sequence.

cAPK recognition sequences, however, are of low complexity and therefore insufficient to pose proteins as specific cAPK substrates. Moreover, in a single cell, cAPK can participate in several parallel pathways that control the phosphorylation status of specific substrates in response to different triggers. Compartmentalization of signaling molecules provides an important level of control to achieve additional signaling specificity. In multicellular organisms, protein kinase A anchor proteins (AKAPs) have been identified that target cAPK holoenzymes to specific subcellular locations (for a recent review, see Ref. 1 and references therein). These AKAPs function as adapters; one domain associates with an AKAP-binding surface created by dimerization of the R-subunit, whereas another distinct domain interacts with a cellular structure or organelle. AKAP-mediated targeting of cAPK is thought to confer spatio-temporal control of cAPK signaling in order to phosphorylate substrates specifically.

In the unicellular eukaryote, Saccharomyces cerevisiae cAPK is responsive to fermentable carbon sources. The addition of glucose or sucrose to cells growing on a nonfermentable carbon source results in a transient increase in the cAMP level (2) and thus (supposedly) of cAPK activity. cAPK controls a multitude of processes involved in growth, metabolism, cell cycle progression, aging, differentiation, and stress resistance (reviewed in Ref. 3). Considering the wide variety of cAPK-controlled effectors in one single cell, it seems likely that subcellular targeting of cAPK, like in multicellular organisms, may contribute to signal specificity.

In budding yeast, localization of cAPK depends on the growth conditions (4, 5). In cells growing rapidly on glucose, the cAPK holoenzyme is found almost exclusively nuclear, whereas, in respiring or in stationary phase cells, Bcy1 is more evenly distributed over both nuclear and cytoplasmic compartments. Phosphorylation of serine residues organized in two different clusters in its N-terminal localization domain are required for cytoplasmic localization of Bcy1 in glucose-starved cells. These phosphorylations appear to be dependent to a large extent on Yak1, a protein kinase whose localization is regulated by glucose availability (6).

Although yeast cAPKs exhibit a strong evolutionary conservation, no classical AKAPs have been identified so far in unicellular organisms (recently discussed in Ref. 7). In budding yeast, however, Zds1 was found to have properties that are reminiscent of AKAPs. First, in a two-hybrid approach, Zds1 was shown to interact with the localization domain of Bcy1, and second, Zds1 appeared to affect the localization of cAPK in glucose-deprived cells (4). Although these data imply that Zds1...
is a functional AKAP homologue, no physiologically relevant function of a presumptive Zds1-Bcy1 interaction has been reported, and this is one of the topics of the present study. Zds1 and its partially redundant homologue Zds2 have been isolated in numerous genetic screens, reflecting diverse roles in the cell. Perhaps the best characterized function so far is their role in numerous genetic screens, reflecting diverse roles in the cell.

Increased Localization, Phosphorylation, and Expression of Bcy1 in Cells Shifted to a Higher Temperature—Previous studies revealed a carbon source-dependent localization pattern of Bcy1 (4, 5). We further extended these studies and demon-

**EXPERIMENTAL PROCEDURES**

**Growth Media, Growth Conditions, Yeast Strains, and Plasmids—** Yeast media were prepared as described (27). Cells were grown in YPAD medium or in synthetic complete (SC) medium supplemented with adenine, uracil, and amino acids as appropriate but lacking essential components to select for plasmids. Yeast strains in this study are listed in Table I. All plasmids used in this study are listed in Table II. All Bcy1 alleles encoding Bcy1 versions bearing substitutions of the serine residues were described previously (4). 313pBHBwt was created by subcloning the plasmids described in Ref. 4 in 313pBHB using NotI and EcoRI. 181pBHB was created by subcloning GFP-HA-BCY1 from 313GHB wt (5) in Yeplac181 using SacI and EcoRI. 181pBHB was created similarly as described above for the 313pBHB derivatives. 112pADH-ZDS1 was created by subcloning the pADH1-ZDS1 fragment from 316pADH-ZDS1 (4) in Yeplac112 using PovII.

**Western Blot Analysis—** Yeast cell cultures were grown at the indicated temperatures (see "Results"). All subsequent steps were carried out at 4 °C. Cells were harvested by centrifugation and washed in sterile water, and the pellets were resuspended in extraction buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 10% glycerol, 5 mM EDTA, 5 mM NaF, 1 mM dithiothreitol, 1 mM EGTA, and a mixture of protease inhibitors (Complete, Roche Applied Science). Cells were disrupted by vortexing for 5 min in the presence of glass beads. The resulting suspension was spun down in a microcentrifuge at maximum speed, and part of the resulting supernatant was taken up in loading buffer, fractionated by SDS-PAGE (28), and blotted onto nitrocellulose. The running gel for separation of Bcy1 phosphoisoforms was buffered with 0.19% Triton HCl.

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**Biochemical Determinations—** The yeast cAPK antagonizes stress-responsive signaling. However, little is known about a presumptive connection between cAPK and stress-controlled pathways. In this work, we provide data pointing to a cross-talk between mitogen-activated protein kinase (MAPK) Slt2/Mpk1 and cAPK. Slt2/Mpk1 is activated by conditions that affect cell wall stability (e.g. heat stress) and is essential for remodeling of the cell wall in order to maintain proper cell shape and integrity (15, 17). Accordingly, mutants defective in Slt2/Mpk1 activation undergo cell lysis, particularly at elevated temperatures. This effect can be prevented by osmotic stabilization indicating inappropriate cell wall biogenesis in such mutants. Upstream of Slt2/Mpk1, cell surface sensors translate cell wall disturbances into activation of Pkc1 kinase (18). Pkc1, in turn, activates a linear MAPK cascade, composed of Bck1 (19, 20), a pair of redundant MAPK kinases (Mkk1 and Mkk2) (21), and finally MAPK Slt2/Mpk1 (22). Downstream effectors of Slt2/Mpk1 are Rim1 (23) and SBF1 (Swi4/Swi6) (24), two transcription factors that activate genes involved in cell wall biogenesis (25) and cell cycle progression, respectively. Moreover, expression of MCK1, encoding a yeast homologue of mammalian glycogen synthase 3 (GSK3), is induced by Slt2/Mpk1 (26). Remarkably, these studies indicated an interaction between yeast Gsk3 signaling and Zds1. Deletion of SLT2/MPK1, BCK1, or MCK1 in a background were ZDS1 was deleted suppresses an otherwise severe G2 delay of the cell cycle.

**RESULTS**

**Increased Localization, Phosphorylation, and Expression of Bcy1 in Cells Shifted to a Higher Temperature—** Previous studies revealed a carbon source-dependent localization pattern of Bcy1 (4, 5). We further extended these studies and demon-
strated that also a rise in temperature from 30 to 37 °C caused a similar response. Whereas at 30 °C GFP-Bcy1wt was found almost exclusively in the nucleus, transfer of the cells to 37 °C led to a slow and progressive increase in the cytoplasmic compartment (Fig. 1A). Quantification of this effect showed that cytoplasmic GFP-Bcy1wt is detectable within 15 min and

![Table II](text)

**TABLE II**

**Plasmids used in this study**

| Plasmid               | Description |
|-----------------------|-------------|
| 313pBHB<sub>wt</sub> | Expresses a HA-BCY1 fusion, encoding HA-tagged wild type Bcy1p, using the BCY1 promoter in plasmid 313pBHB<sub>wt</sub> in Ref. 4. |
| 313pBHB<sub>S3A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S4A, and S9A substitutions. |
| 313pBHB<sub>S4A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S74A, S77A, S79A, S81A, S83A, and S84A substitutions. |
| 313pBHB<sub>S3S4A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S4A, S9A, S74A, S77A, S79A, S81A, S83A, and S84A substitutions. |
| 313pBHB<sub>S74A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 313pBHB<sub>S77A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 313pBHB<sub>S79A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 313pBHB<sub>S81A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 313pBHB<sub>S83A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 313pBHB<sub>S84A</sub> | 313pBHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S9A, S74D, S77D, S79D, S81D, S83D, and S84D substitutions. |
| 181pBGHB<sub>wt</sub> | Expresses a GFP-HA-BCY1 fusion, encoding HA-tagged wild type Bcy1p, using the BCY1 promoter in YEplac181 (51). |
| 181pBGHB<sub>S3A</sub> | 181pBGHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S4A, and S9A substitutions. |
| 181pBGHB<sub>S4A</sub> | 181pBGHB<sub>wt</sub> derivative encoding Bcy1 bearing S74A, S77A, S79A, S81A, S83A, and S84A substitutions. |
| 181pBGHB<sub>S3S4A</sub> | 181pBGHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S4A, S9A, S74A, S77A, S79A, S81A, S83A, and S84A substitutions. |
| 33A2-pBHB<sub>wt</sub> | Expresses a HA-BCY1 fusion, encoding HA-tagged wild type Bcy1p, using the BCY1 promoter in vector 33A2 (CEN, ADE2, ARS, this study) |
| 195A2-pBGHB<sub>wt</sub> | Expresses a GFP-HA-BCY1 fusion, encoding HA-tagged wild type Bcy1p, using the BCY1 promoter in 195A2 (4). |
| 195A2-pBGHB<sub>S3S4A</sub> | 195A2-pBGHB<sub>wt</sub> derivative encoding Bcy1 bearing S3A, S4A, S9A, S74A, S77A, S79A, S81A, S83A, and S84A substitutions. |
| 112pADH1-ZDS1 | Expresses ZDS1, using the ADH1 promoter, in vector YEplac112 (51). |
| YC plac33 | Expresses a dominant active allele of BCK1 (19). |
| YEplac112 | 2μm, TRP1 vector (51). |

**Fig. 1.** Kinetics of localization and phosphorylation properties of Bcy1 after transfer from 30 to 37 °C. A, fluorescence microscopy of W303-1A cells transformed with plasmid 195A2-pBGHB<sub>wt</sub>. Pictures were taken of cells that were growing logarithmically on YPD at 30 °C (0') and subsequently 60 and 180 min after transfer to 37 °C. B, quantification of GFP-Bcy1<sub>wt</sub> localization shown in Fig. 1A. The percentage of cells with detectable GFP-Bcy1<sub>wt</sub> in the cytoplasm has been determined as a function of time. C, Western analysis of extracts isolated from MR1 (bcy1) cells transformed with plasmid 313pBHB<sub>wt</sub> that were growing logarithmically on YPD at 30 °C (0') and subsequently transferred to 37 °C at the indicated times. As a loading control, the level of alcohol dehydrogenase was determined. D, protein extracts of the 0- and 120-min sample shown in Fig. 1C were treated with phosphatase prior to Western analysis. PPase, λ-phosphatase.
reaches a maximum 2 h after the shift to 37 °C (Fig. 1B). Subsequently, the phosphorylation status of Bcy1 after transfer from 30 to 37 °C was determined. Western analysis of heat-stressed cells producing a HA-tagged version of Bcy1 revealed that, at 37 °C, the presence of slower migrating forms of Bcy1 was most dominantly present after about 60–120 min (Fig. 1C). Phosphatase treatment prior to Western analysis resulted for unknown reasons in a faster mobility relative to wild type Bcy1 irrespective of the temperature and thus clearly not the consequence of temperature-dependent phosphorylation differences. Nonetheless, all isoforms detectable at 37 °C migrated (unlike wild type Bcy1) with equal or faster mobility compared with the situation at 30 °C. Moreover, all isoforms detected at 37 °C reached a maximum 2 h after the shift to 37 °C (Fig. 1B). Subsequently, the phosphorylation status of Bcy1 after transfer from 30 to 37 °C was determined. Western analysis of extracts isolated from (MR1) bcy1 cells transformed with plasmids 313pBHB wt, 313pBHB(S cI A), 313pBHB(S cII A), or 313pBHB(S cI D) that were growing logarithmically on YPD at 30 °C (0°) and subsequently transferred for 120 min at 37 °C. The middle panel is a longer exposure of the upper panel in order to better visualize the weaker bands. As a loading control, the level of alcohol dehydrogenase was determined. B, Western analysis of extracts isolated from W303-1A cells transformed with plasmids 313pBHB wt, 313pBHB(S cI A), 313pBHB(S cII A), or 313pBHB(S cI D) that were growing logarithmically on YPD at 30 °C. C, fluorescence microscopy of W303-1A cells transformed with plasmid 181pBGHB wt or 181pBGHB(S cI A) that were growing on YPD at 37 °C. D, quantification of the localization patterns in W303-1A cells transformed with 181pBGHB wt, 181pBGHB(S cI A), 181pBGHB(S cII A), or 181pBGHB(S cI D) that were grown at 37 °C. The mean percentage of cells with a more intense nuclear fluorescence relative to cytoplasmic fluorescence was determined. Three independent transformants were assayed at least three times each (at least 100 cells counted for each determination). Error bars, S.D. DAPI, 4,6-diamidino-2-phenylindol.

Serine Residues Located in the Bcy1 Targeting Domain Are Required for Heat Stress-induced Phosphorylation and Consequently for Efficient Expression and Cytoplasmic Localization—In previous studies, two serine-rich clusters in the Bcy1 localization domain were identified that are required for carbon source-dependent phosphorylation. To test whether these clusters are also subjected to heat stress-induced phosphorylation, we studied migration, expression, and localization of Bcy1 versions bearing substitutions of these residues to alanines. Western analysis showed that replacement of cluster I serines to alanines (Bcy1(S cI A)) reduced to a large extent the formation of slower migrating isoforms (upper and middle panels of Fig. 2A). Remarkably, substitution of cluster II serines (Bcy1(S cII A)) resulted for unknown reasons in a faster mobility relative to wild type Bcy1 irrespective of the temperature and thus clearly not the consequence of temperature-dependent phosphorylation differences. Nonetheless, all isoforms detectable at 37 °C migrated (unlike wild type Bcy1) with equal or faster mobility compared with the situation at 30 °C, although the effects are undoubtedly less pronounced compared with the cluster I replacements. Finally, Bcy1(S cI cII A) displayed a dramatically increased migration relative to wild type. Altogether, we conclude that both serine-rich clusters are required for proper Bcy1 phosphorylation after heat stress. Note that even in the case when both clusters of serines are substituted, an isoform was still detectable, possibly representing autophosphorylation of Ser145 (32). These experiments also revealed that the intracellular levels of Bcy1(S cI A) and Bcy1(S cII A) were extremely low, raising the possibility that phosphorylation of the serines comprising cluster I are important for proper expression of Bcy1. To gain more support for this hypothesis, we studied whether mimicking constitutive phosphorylation by introduction of negatively charged residues (aspartic acid) at these positions is sufficient to obtain normal levels of intracellular Bcy1. Western analysis revealed that the levels of HA-Bcy1(S c I D) and HA-Bcy1(S c II D)
but not of Bcy1(S cI A) and Bcy1(S cI /H11001 cII A), were similar to HA-Bcy1wt (Fig. 2B), indeed suggesting that phosphorylation of the corresponding serines is required for appropriate expression of BCY1. To determine whether these phosphorylations affect the subcellular localization, we performed fluorescence microscopy of yeast cells producing these Bcy1 substitution mutants fused with GFP (Fig. 2C). This analysis revealed that replacement of the serines comprising both clusters with alanines led to a relatively high nuclear localization at 37°C. As has been noted before (4), the subcellular distribution of GFP-Bcy1 in different cells in a culture is not uniform. We therefore quantified the effects on localization of individual cells of each culture (Fig. 2D). This showed that both clusters independently promote cytoplasmic localization.

In conclusion, both clusters of serines are subjected to heat stress-instigated phosphorylation, and these phosphorylations are required for efficient cytoplasmic localization. Moreover, phosphorylation of cluster I serines affects expression of Bcy1. Note that expression of Bcy1(S cI A) is also low at 30°C compared with wild type Bcy1, indicating that even in unstressed cells at least some phosphorylation seems to be required for maintaining appropriate levels of Bcy1.

**Yeast GSK3s Are Required for Temperature-dependent Phosphorylation of Bcy1**—In order to gain more insight into the
molecular mechanism of heat-induced phosphorylation and cytoplasmic localization of Bcy1, we followed these parameters in yeast mutants bearing one or more deletions of genes that encode kinases. In previous studies, Yak1 kinase was found to be required for cytoplasmic localization of Bcy1 in glucose-deprived cells. However, in yak1 cells grown at 37°C, cytoplasmic targeting of GFP-Bcy1 wt was not affected detectably, suggesting that this kinase is not important for temperature-dependent Bcy1 localization. In vitro studies (33) demonstrated that GSK3 can phosphorylate RII, the mammalian homologue of Bcy1. In yeast, four closely related genes, RIM11, MCK1, MRK1, and open reading frame YOL128c (we propose to name open reading frame YOL128c as YGK3 (for yeast homologue of glycogen synthase kinase 3) have been identified that encode yeast homologues of mammalian GSK3 (34). As a first semi-quantitative test of whether yeast GSK3 might control phosphorylation (and consequently localization) of Bcy1, we determined the subcellular distribution of GFP-Bcy1 wt at 30 and 37°C in gsk3 mutants. As expected, microscopic analysis revealed that GFP-Bcy1 is almost exclusively nuclear in both wild type and gsk3 mutant cells when grown at 30°C (data not shown). At 37°C, however, in mck1, mrk1, and ygk3, but not in rim11 cells, GFP-Bcy1 wt remained more concentrated in the nucleus compared with wild type cells. (Fig. 3, A and B). No additive effect on the extent of nuclear accumulation was detectable in this assay in the double or triple gsk3 mutant cells. However, it should be noted that a microscopic analysis such as performed here does not allow a comparison of the GFP-Bcy1 levels in cytoplasm or nucleus between the different mutants, although BCY1 expression might also be dependent on phosphorylation (Fig. 2). These data revealed that Mck1, Mrk1, and Ygk3, but not Rim11, are required (in a redundant fashion) for cytoplasmic localization of GFP-Bcy1 in heat-stressed cells, thus raising the possibility that these kinases play a role in Bcy1 phosphorylation.

To study more directly a function of Mck1, Mrk1, and Ygk3 in Bcy1 phosphorylation, we followed HA-Bcy1 wt mobility in the corresponding gsk3 mutants before and after heat shock (Fig. 3C). Western analysis revealed that some of the slower migrating heat stress-induced isoforms of HA-Bcy1 wt are absent in mck1 cells. This effect, although somewhat less pronounced, is also observed when using an mrk1 ygk3 deletion strain (in the corresponding single mutants no migration differences could be observed; data not shown). In the triple gsk3 deletion strain, mobility of HA-Bcy1 wt appeared to be most dramatically affected. These results show that Mck1, Mrk1, and Ygk3 are required to various degrees for Bcy1 phosphorylation after heat shock. Mck1, however, appears to be most prominently involved in this process. It should be mentioned that even in the mck1 mrk1 ygk3 strain, modification is not completely absent, indicating that kinases other than yeast GSK3 may also promote phosphorylation at 37°C.

Previous studies indicated that Slt2/Mpk1 upon activation by Ca²⁺ leads to increased Mck1 activity (26). Since Mck1 is required for heat-activated phosphorylation of Bcy1, we studied whether the addition of Ca²⁺ is sufficient for Bcy1 phosphorylation and to what extent this depends on Mck1, Mrk1, and Ygk3. Western analysis of extracts from gsk3 strains...
showed that the addition of 0.15 M CaCl₂ led to the formation of slower migrating HA-Bcy1wt, and this effect appears to require mainly Mck1 (Fig. 3D). This result further corroborates a function of this kinase in Bcy1 phosphorylation. In the mck1ygk3 strain, no mobility differences are observed, excluding them from a dominant role in Ca²⁺/H⁺-instigated modification of Bcy1.

We addressed the question of whether defective Bcy1 phosphorylation in gsk3 mutants after heat shock is the primary cause of its reduced cytoplasmic recruitment or alternatively an indirect effect elicited by the deletion of the respective genes. To study these possibilities, we determined the localization of a GFP-tagged version of Bcy1 with both clusters of serines substituted for aspartate (GFP-Bcy1(S₁₁₁/₁₂₂ A)) in mck1 mrrk1 ygk3 cells (Fig. 3E). Fluorescence microscopy revealed that, unlike GFP-Bcy1wt, GFP-Bcy1(S₁₁₁/₁₂₂ A) was found evenly distributed over the nucleus and cytoplasm, indicating that the relatively...
low levels of cytoplasmic Bcy1 after heat stress in yeast gsk3 mutants is a direct consequence of compromised Bcy1 phosphorylation.

Yeast gsk3 Cells Are Sensitive to Cell Wall Stress—Mek1 acts as a downstream effector of Slt2/Mpk1 (26), and deletion of the gene was shown to result in heat sensitivity (35) (Fig. 4A). However, mrk1, ykg3, and even mrk1 ykg3 cells are not heat-sensitive (Fig. 4A), indicating a less prominent role of these kinases in the response to heat stress compared with Mek1.

Cell wall stress can also be generated by the addition of an anionic detergent (36). In the presence of 0.01% SDS, which is expected to cause a severe cell wall stress, not only mck1 but also mrk1 and ykg3 cells are unable to grow (Fig. 4A). We also tested the sensitivity of gsk3 mutants to zymolase (β-1,3-glucanase) and calcofluor white, two cell wall stress-causing agents that digest or bind to cell wall polymers, respectively (Fig. 4B). In the presence of these agents, the growth of mck1, mrk1, and ykg3 cells was severely reduced. The effects on the mrk1 ykg3 and mck1 mrk1 ykg3 mutant were even more pronounced, indicating that these kinases act redundantly. Since the growth rate of wild type and gsk3 mutants is virtually identical (data not shown), the reduced growth in the presence of cell wall stressing agents is not an indirect side effect of a general growth defect.

Altogether, these data indicate that all three kinases are to various degrees responsive (presumably activated) to cell wall stress in order to survive under such conditions. In agreement with this notion, Western analysis revealed that upon the addition of SDS to the medium, slower migrating isoforms of Bcy1 were produced (results not shown).

Heat Stress-induced Phosphorylation of Bcy1 Does Not Affect cAPK Catalytic Activity—To study the physiological relevance of Bcy1 phosphorylation and its consequences on localization and expression, several parameters known to be regulated by cAPK activity were studied. In these studies, yeast mutants produced versions of Bcy1 with substitutions of the serines comprising cluster I and/or II to alanines (and therefore compromised in heat-stress-induced phosphorylation) (Fig. 2). When introduced in a bcy1 strain, these mutant alleles of BCY1 complemented the growth defect of such a strain on a nonfermentable carbon source (Fig. 5A).

The activity of the general stress response element is strongly repressed by cAPK (37). However, heat stress-induced activation of a stress response element-driven reporter gene β-galactosidase was not affected (Fig. 5B). We also measured trehalase activity from extracts of these mutant strains. Since trehalase (Nth1) is a cytoplasmic localized enzyme and is activated by cAPK phosphorylation (38), we reasoned that this enzyme could be an excellent example of a cAPK substrate potentially regulated by differential localization of Bcy1. However, in extracts from the respective Bcy1 cluster mutants, trehalase activity was not affected significantly (Fig. 5C). We excluded the possibility that the Nth1 activity is held at an equal level among the different strains by a possible compensatory mechanism at the level of NTH1 expression, since the levels of Nth1 were found to be similar among the different strains (Fig. 5D). Note that Nth1 levels are increased after heat shock, presumably by virtue of functional stress response elements in the NTH1 promoter (39). Finally, trehalase and glycogen levels are exquisitely sensitive to changes in cAPK activity. However, heat stress-induced trehalase and glycogen accumulation was not affected significantly by the Bcy1 substitutions (Fig. 5, E and F). Collectively, these results show that several well known cAPK-controlled processes are insensitive to phosphorylation of Bcy1. We conclude that these stress-induced modifications do not seem to affect its capacity to inhibit the cAPK catalytic subunits. Apparently, the altered distribution of Bcy1 over nucleus and cytoplasm as a consequence of these mutations is not important for cAPK-controlled activities (at least those measured here). Even the dramatically reduced expression of HA-Bcy1S1,2Δ and HA-Bcy1S1,2Δ,Δ was not found important for any of the processes studied here.

Cytoplasmic Recruitment of Bcy1 in Heat-stressed Cells Depends on Zds1 and Zds2—The results of Fig. 5 imply that heat stress-induced phosphorylation of Bcy1 and its effects on localization and expression do not change the overall cAPK catalytic activity in the cell, thus pointing to another distinct function of Bcy1 apart from inhibiting the cAPK subunits. We hypothesized that the in vivo role of phosphorylation is to recruit Bcy1 to one or more specific cAPK substrates that are presumably localized in the cytoplasm. In this way, cAPK-mediated regulation of these (perhaps novel) targets is dependent on phosphorylation of Bcy1. Two proteins that may be involved in such a cytoplasmic recruitment are Zds1 and Zds2, two candidates for functional AKAP homologues that are essential for efficient cytoplasmic localization of Bcy1 in respiratory and stationary phase cells (4). Fluorescence microscopy revealed that indeed in zds1 and zds2 cells at 37 °C, GFP-Bcy1wt remained more concentrated in the nucleus compared with wild type cells (Fig. 6), demonstrating that also in heat-stressed cells Zds1 and Zds2 are required for proper cytoplasmic targeting of Bcy1.

Hyperactive GSK3 Signaling in zds1 Cells—A possible explanation for the lowered cytoplasmic recruitment of GFP-Bcy1wt in zds1 and zds2 cells at 37 °C is that in such mutants Bcy1 phosphorylation is diminished. Western analysis, however, revealed that HA-Bcy1wt from zds1 cells migrated more slowly already at 30 °C (Fig. 7A). This unexpected result suggests that signaling toward Bcy1 phosphorylation is constitutively hyperactive in zds1 cells. Since heat stress-induced phosphorylation of Bcy1 depends on yeast GSK3 (Fig. 3), we studied whether the constitutive modification of Bcy1 in zds1 cells also requires GSK3. To address this question, strains were constructed with MCK1 or MCK1 MRK1 and YGK3 deleted in a zds1 background. Analysis of the HA-Bcy1wt migration patterns (Fig. 7B) demonstrated that the slower migrating isoforms of HA-Bcy1wt in zds1 cells are already absent when only MCK1 is deleted indicating that (at least) Mck1 is hyperactive in these mutants. Since MAPK Slt2/Mpk1 acts upstream of Mck1, we determined whether activity of Slt2/Mpk1 is dependent on Zds1. Activation of MAPKs requires dual phosphorylation of a conserved TXY motif (40). We measured the Slt2/Mpk1 phosphorylation status
by using an antibody that specifically recognizes dual phosphorylation of the corresponding Thr\textsuperscript{190} and Tyr\textsuperscript{192} residues (41). Western analysis revealed that in \textit{zds1} cells, the levels of dually phosphorylated Slt2/Mpk1 (and presumably of total Slt2/Mpk1 kinase activity) are higher (Fig. 7\textit{C}). However, subsequent detection (in the same lanes) of total Slt2/Mpk1 also revealed a higher expression of \textit{SLT2}/\textit{MPK1} that may, at least partially, be responsible for the elevated levels of phospho-Slt2/Mpk1. Note that Slt2/Mpk1 activation and expression constitute an autoregulatory loop, since Slt2/Mpk1 expression is dependent on Rlm1 (25), a transcription factor activated by Slt2/Mpk1, and could provide a plausible explanation for the higher expression of \textit{SLT2}/\textit{MPK1} in \textit{zds1} cells.

Overexpression of \textit{ZDS1} Affects Cell Wall Integrity at Elevated Temperatures—The results from Fig. 7\textit{C} suggest that Zds1 may act as a negative regulator of Slt2/Mpk1 activity. We reasoned that overproduction of Zds1, when functioning as a repressor of Slt2/Mpk1, may render the cells thermosensitive. Indeed, overexpression of \textit{ZDS1} in wild type cells resulted in a temperature-dependent growth defect (Fig. 7\textit{D}). Importantly, this effect could be suppressed by the addition of 1 M sorbitol in the medium, indicating that elevated levels of Zds1 production affect cell wall integrity.

Remarkably, cells that overexpress \textit{ZDS1} did not show an increased sensitivity to zymolyase or calcofluor (data not shown), suggesting that the observed effects of Zds1 seem more specific for heat stress rather than for cell wall stresses in general.

\textit{Bcy1(S\textsubscript{cI/H11001cII D})} Decreases Cell Wall Integrity, and This Effect Is Dependent on \textit{Zds1} and \textit{SLT2}/\textit{MPK1}—Above we presented evidence that Zds1 is required for cytoplasmic recruitment of phosphorylated Bcy1 in heat-stressed cells (Fig. 6). Since Zds1 affects cell wall integrity signaling (Fig. 7), we studied possible effects of Bcy1 phosphorylation on the Zds1-induced thermosensitivity. In these experiments, we used strains that produced a mutant version of Bcy1 bearing substitutions of the cluster serines to aspartate (Fig. 8\textit{A}). These substitutions appear to mimic constitutive phosphorylation of the corresponding serine residues well, since these were shown to confer a constitutive nucleocytoplasmic localization and a dramatically reduced migration on SDS-PAGE (Figs. 2\textit{B} and 3\textit{D}).

HA-Bcy1\textsubscript{S\textsubscript{cI/H11001cII D}} produced in wild type cells did not have a
detectable effect on growth at higher temperatures (data not shown). However, co-overexpression of ZDS1 in such cells dramatically exacerbated the growth defect caused by Zds1 overproduction alone and is in fact only partially suppressed by the addition of 1 M sorbitol in the medium (Fig. 8A). Co-transformation of this strain with a plasmid carrying a dominant active allele of BCK1 (BCK1–20) suppresses the growth defect fully at 38.5 °C but only partially at 40 °C. Full growth at 40 °C is only achieved when 1 M sorbitol is added to the medium (Fig. 8A).

Altogether, these data suggest that Bcy1 can affect cell wall integrity depending on Zds1 and on the phosphorylation status of the cluster serines. Since these effects are suppressed by sorbitol and/or by introduction of BCK1–20, we conclude that the observed thermosensitivity is (at least to some extent) the consequence of diminished Slt2/Mpk1 activation. Note that the effects of HA-Bcy1(S cI/H11001 cII D) are dominant and thus unlikely to be the result of reduced inhibition of the cAPK catalytic subunits.

Increased Levels of Dually Phosphorylated Slt2/Mpk1 in Yeast GSK3 Mutants—The results of Fig. 8A suggest that phosphorylation of Bcy1 can down-regulate signaling through Slt2/Mpk1. Since Bcy1 phosphorylation in response to heat stress requires yeast GSK3, we studied dual Slt2/Mpk1 phosphorylation in several yeast gsk3 mutants after transfer to elevated temperature (Fig. 8B). Western analysis revealed that in mck1 cells, a relatively high Slt2/Mpk1 phosphorylation was observed after 120 min at 37 °C. A weak increase was observed in extracts from an mrk1 ygk3 strain. Since the levels of total Slt2/Mpk1 are comparable between the different strains, we conclude that signaling toward Slt2/Mpk1 phosphorylation of Thr190 and Tyr192 is higher in gsk3 cells after heat stress.

**DISCUSSION**

**Yeast GSK3 Is Required for Heat Stress-instigated Phosphorylation of Bcy1**—Here we present evidence demonstrating an unprecedented responsiveness of cAPK to heat stress. A mild heat shock to 37 °C triggers a relatively slow but progressive subcellular redistribution of its regulatory subunit Bcy1 that culminates in a more even localization over nucleus and cytoplasm relative to the situation at 30 °C. This temperature-dependent relocalization is controlled by phosphorylation of serine residues present in its N-terminal localization domain. We propose that specific Bcy1 localization in response to environmental cues imposes an additional regulatory level on yeast cAPK that may help to ensure phosphorylation of bona fide substrates under particular conditions.

Apart from the effects on subcellular redistribution, heat stress also leads to an increase of the Bcy1 levels. This effect involves also phosphorylation of its N-terminal domain, but only of the serines comprising cluster I. Increased expression of
Bcy1 has been reported before in cells grown to stationary phase (32). In such cells, Bcy1 is also distributed over the nucleocyttoplasm, and its localization domain is hyperphosphorylated (4). Possibly, control of its expression might be important to maintain a certain minimal threshold concentration in stressed cells (i.e., at 37 °C or in stationary phase), since under such conditions Bcy1 is distributed in a much larger volume (nucleus and cytoplasm) compared with unstressed cells (nucleus).

Our studies presented here implicate Bcy1 as a target of the Pck1-Slt2/Mpk1 pathway. Activation of Slt2/Mpk1 by heat stress or Ca\(^{2+}\) led to increased phosphorylation of Bcy1. These phosphorylations were dependent mainly on Mck1 (a known downstream target of Slt2/Mpk1) and to a lesser extent on two other GSK3 homologues Mrk1 and Ygk3. It remains to be established whether yeast Gsk3 phosphorlates Bcy1 directly or whether it controls the activity of other signaling pathways that ultimately affect the phosphorylation status of Bcy1 indirectly. We deem the first possibility likely because cluster II serines comprise several Gsk3 consensus sites (7). Moreover, mammalian RII subunits can be phosphorylated in vitro by purified Gsk3 (33), suggesting that phosphorylations on cAPKs are evolutionarily conserved.

Apart from controlling Bcy1 phosphorylation in response to heat stress, we observed that mrk1 and ygk3 cells are sensitive to agents that elicit cell wall stress (Fig. 4), indicating that (like Mck1) Mrk1 and Ygk3 also play an role in cell wall integrity signaling.

**Phosphorylation of Bcy1 May Activate Zds1, a Negative Regulator of Cell Wall Integrity Signaling**—These and other studies (4) revealed that differential phosphorylation of Bcy1 affects its intracellular localization. Bcy1 phosphorylation, however, is not sufficient for cytoplasmic localization but requires also Zds1 and Zds2, two putative yeast AKAPs that may associate with phosphoisoforms of Bcy1 (4). Contrary to the situation in yeast cells, in multicellular organisms, it is generally assumed that R-subunits associate tightly to their corresponding AKAPs, resulting in a fixed subcellular localization (1). However, in mammalian cells a cell cycle-dependent control of R-subunit localization has recently been reported. These studies indicated that binding of cAPK to AKAPs is controlled by phosphorylation of the R subunit localization domain (42, 43). Cyclin B-p34\(^{cd2}\)-dependent phosphorylation of RII was found to reduce affinity for AKAP450 but to increase it for AKAP95, providing an explanation for the observed dynamics of RII localization during the cell cycle. Collectively, control of R-subunit binding to AKAPs by phosphorylation of its localization domain may constitute a general mechanism for differential cAPK localization in response to intra- or extracellular triggers.

What is the in vivo function of the heat stress-induced effects on phosphorylation and its consequences for Bcy1 localization and expression? Classical processes that are known to be controlled by cAPK were found unaffected (Fig. 5), even those that are presumed exclusively cytoplasmic. This led us to conclude that Bcy1 phosphorylation and its consequences on localization and expression do not affect its capacity to inhibit (local pools of) the cAPK catalytic subunits.

An alternative model, more analogous to the situation in other eukaryotes, is that phosphorylation of Bcy1 recruits the cAPK holoenzyme to specific cytoplasmic localized substrates possibly by an interaction with Zds1 and Zds2. In this way, activities of such presumptive cytoplasmic targets could be controlled by cAPK, depending on Zds1 and Zds2 and the phosphorylation status of Bcy1. Thus, a crucial question concerns the in vivo function of Zds1 and Zds2 and whether such a function is regulated by cAPK phosphorylation.

Here we show that in zds1 cells the level of dually phosphorylated Slt2/Mpk1 is constitutively high and that as a likely consequence, Bcy1 is found hyperphosphorylated (in an Mck1-dependent manner) in such cells already at 30 °C. Moreover, ZDS1 overexpression resulted in decreased cell wall integrity at elevated temperatures. These data imply that Zds1 acts as a negative regulator of Slt2/Mpk1 activity. Consistent with this idea, several other processes that are dependent on Slt2/Mpk1 (such as high Swe1 activity, a prolonged G2 phase of the cell cycle, sensitivity to Ca\(^{2+}\), and hyperpolarized growth) are hyperstimulated in zds1 cells, and these effects are suppressed by additional deletion of SLT2/MPK1, BCK1, or MCK1 (8, 26). The molecular mechanism of this presumptive Slt2/Mpk1 inhibition by Zds1 is currently unknown and requires further studies. It is noteworthy that in neurons AKAP79 binds and thereby inhibits protein kinase C directly (44). In yeast, Zds2 (a close homologue of Zds1) was found to interact with protein kinase C (45), thus providing an evolutionarily conserved concept of how Zds1 may control Slt2/Mpk1 activity.

Heat-stress-instigated phosphorylation of the Bcy1 localization domain may trigger Zds1-dependent inhibition of Slt2/Mpk1. We show that the effects of ZDS1 overexpression on thermosensitivity are dramatically enhanced in the presence of a version of Bcy1 that mimics a constitutively phosphorylated form. These effects are suppressed by (artificial) activation of Slt2/Mpk1 and/or by the addition of 1 M sorbitol in the medium, indicating a reduced cell wall integrity signaling in these strains. Consistent with these interpretations, in gsk3 cells, Slt2/Mpk1 phosphorylation is higher after heat stress. Presumably lowered Bcy1 phosphorylation in these mutants renders Bcy1 a less potent activator of Zds1. The precise molecular mechanism of how Bcy1 and Zds1 control cell integrity signaling remains to be established. Possibly, association of Bcy1 with Zds1 may facilitate cAPK-mediated phosphorylation of components that are involved in cell wall integrity signaling and may point to cross-talk between cAPK and protein kinase C-Slt2/Mpk1 signaling. Alternatively, Zds1 is a cAPK substrate, and phosphorylation would lead to activation of its capacity to down-regulate Mpk1. Although overexpression of ZDS1 leads to increased thermosensitivity, growth in the presence of zymolyase or calcofluor white, agents that also decrease cell wall integrity, is not affected. Apparently, cell wall stress

**FIG. 9. Working model of feedback control of cell wall integrity signaling by Bcy1 and Zds1.** The arrows and the blunt-ended line indicate positive and negative controls, respectively. Activation of the cell wall integrity pathway leads to Slt2/Mpk1-Mck1-mediated phosphorylation of Bcy1. Phosphorylated Bcy1 appears to promote a Zds1-dependent down-regulation of cell wall integrity signaling at a point that has not yet been identified.

Heat Stress-instigated GSK3 Phosphorylation of cAPK
alone is not sufficient to elicit the dominant negative effects of Zds1 overexpression found in heat-stressed cells.

The role of Zds2 is less clear. Like Zds1, Zds2 was also identified as a negative regulator of polarized growth, acting redundantly with Zds1 but with a much lower activity (13). This might be the reason that, although Zds2 may also interact and recruit Bcy1 to the cytoplasm, it does not have a major impact on protein kinase C-Slt2/Mpk1 signaling. Indeed, in zds2 cells (unlike in a zds1 mutant), we did not observe hyper-phosphorylation of Bcy1 (Fig. 7A).

Collectively, the data presented here suggest the existence of a negative feedback control on cell wall integrity signaling (Fig. 9). In this model, Mck1-dependent phosphorylation of Bcy1 would lead to inhibition of the pathway in a Zds1-dependent manner.

It is noteworthy in this context that the Pkc1-Slt2/Mpk1 pathway appears to have several positive feedback loops. For instance, Rlm1, a downstream effector of Mpk1, activates in an (indirect) activator of the pathway (16, 24, 46). According to our unpublished data (2), the activation of Slt2/Mpk1 by cell wall stress would lead to a relatively slow GSK3-mediated phosphorylation of Bcy1 that in turn down-regulates or restrains the pathway in a Zds1-dependent manner (Fig. 9). We hypothesize that such a negative feedback inhibition exerted by phosphorylation of cAPK and Zds1 may help to prevent unbridled activation of Slt2/Mpk1, allowing growth and division as efficiently as possible.

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