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Schizosaccharomyces pombe Hsp90/Git10 Is Required for Glucose/cAMP Signaling

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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* senses environmental glucose through a cAMP-signaling pathway. Elevated cAMP levels activate protein kinase A (PKA) to inhibit transcription of genes involved in sexual development and gluconeogenesis, including the *fbp1*$^+$ gene, which encodes fructose-1,6-bisphosphatase. Glucose-mediated activation of PKA requires the function of nine *g*lucose-*i*nsensitive *t*ranscription (*git*) genes, encoding adenylate cyclase, the PKA catalytic subunit, and seven "upstream" proteins required for glucose-triggered adenylate cyclase activation. We describe the cloning and characterization of the *git10*$^+$ gene, which is identical to *swo1*$^+$ and encodes the *S. pombe* Hsp90 chaperone protein. Glucose repression of *fbp1*$^+$ transcription is impaired by both *git10*$^-$ and *swo1*$^-$ mutant alleles of the *hsp90*$^+$ gene, as well as by chemical inhibition of Hsp90 activity and temperature stress to wild-type cells. Unlike the *swo1*$^-$ mutant alleles, the *git10-201* allele supports cell growth at 37°, while severely reducing glucose repression of an *fbp1-lacZ* reporter, suggesting a separation-of-function defect. Sequence analyses of three *swo1*$^-$ alleles and the one *git10*$^-$ allele indicate that *swo1*$^-$ mutations alter core functional domains of Hsp90, while the *git10*$^-$ mutation affects the Hsp90 central domain involved in client protein binding. These results suggest that Hsp90 plays a specific role in the *S. pombe* glucose/cAMP pathway.

GLUCOSE signaling pathways regulate gene expression in both prokaryotic and eukaryotic cells and have been well studied in a variety of model organisms. The fission yeast *Schizosaccharomyces pombe* monitors glucose to regulate sexual development and metabolism. Our studies focus on the transcriptional regulation of the glucose-repressed *fbp1*$^+$ gene, which encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase (Vassarotti and Friesen 1985). Previously, we identified mutations in genes that confer constitutive *fbp1*$^+$ transcription (Hoffman and Winston 1990). These *g*lucose-*i*nsensitive *t*ranscription (*git*) genes encode the components of a protein kinase A (PKA) pathway (Hoffman 2005b), which acts antagonistically to a stress-activated MAPK (SAPK) pathway required for *fbp1*$^+$ transcription (Stettler *et al.* 1996; Stiefel *et al.* 2004). The *git2*$^+$/*cyr1*$^+$ gene encodes adenylate cyclase (Hoffman and Winston 1991), which produces the second messenger cAMP to activate PKA, whose catalytic subunit is encoded by the *pka1*$^+$/*git6*$^+$ gene (Jin *et al.* 1995) and whose regulatory subunit is encoded by the *cgs1*$^+$ gene (Devoti *et al.* 1991). Seven additional *git* genes are required for adenylate cyclase activation and form at least two functionally distinct groups. Four genes encode the Git3 G-protein-coupled receptor (Welton and Hoffman 2000) and its cognate heterotrimeric G protein composed of the Gpa2 Gα (Isshiki *et al.* 1992; Nocero *et al.* 1994), the Git5 Gβ (Landry *et al.* 2000), and the Git11 Gγ (Landry and Hoffman 2001). The Git3 GPCR and Git5-Git11 Gβγ dimer are required for Gpa2 Gα activation and can be bypassed by mutations that activate Gpa2 (Welton and Hoffman 2000), which directly binds and activates adenylate cyclase (Ivey and Hoffman 2005). The *git1*$^+$, *git7*$^+$, *git10*$^+$ are required for glucose repression of *fbp1*$^+$ transcription, even in a strain carrying the *gpa2*^{R176H} activated allele (Welton and Hoffman 2000). Therefore, Git1, Git7, and Git10 either function independently from Gpa2 to activate adenylate cyclase or are required for Gpa2-mediated activation of adenylate cyclase. Git1 is a C2-domain protein that directly binds adenylate cyclase (Kao *et al.* 2006), while Git7 (Schadick *et al.* 2002) is a member of the Sgt1 protein family, whose *Saccharomyces cerevisiae* ortholog has been implicated in both adenylate cyclase function (Dubacq *et al.* 2002) and kinetochore assembly (Kitagawa *et al.* 1999).

We describe here the cloning of the *git10*$^+$ gene, which is identical to the previously identified *swo1*$^+$ gene encoding the only *S. pombe* Hsp90 heat-shock chaperone protein. (For clarity, we refer to the gene as *hsp90*$^+$ and to mutant alleles as either *swo1*$^-$ or *git10*$^-$ alleles of *hsp90*$^+$.) The *git10*$^-$ and *swo1*$^-$ alleles confer some overlapping phenotypes; however, the *git10-201* allele is more limited to causing a severe cAMP-signaling defect without the

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temperature-sensitive growth defects associated with *swo1*⁻ alleles. Hsp90 activity is required for proper glucose/cAMP signaling as both prolonged heat stress and Hsp90 inhibition by geldanamycin increase expression of an *fbp1-lacZ* reporter. As *git10-201* strains display a significant defect in *fbp1-lacZ* regulation even at 25°, while remaining viable at 37°, it appears that *git10-201* is a separation-of-function mutation specifically affecting Hsp90 function in the cAMP pathway. Consistent with this hypothesis, the *git10-201* mutation alters a residue in the central domain of the protein, which is presumably involved in client protein binding, while the mutations in three *swo1*⁻ mutant alleles alter residues in the N-terminal ATP-binding domain or the C-terminal dimerization domain. Thus, there appears to be a direct role for Hsp90 in the *S. pombe* glucose/cAMP pathway, and it is not just that mutations in the *hsp90*⁺ gene confer a general stress signal to derepress *fbp1*⁺ transcription.

MATERIALS AND METHODS

***S. pombe* strains and growth media:** Yeast strains used in this study are listed in Table 1. The *fbp1::ura4*⁺ and *ura4::fbp1-lacZ* reporters are translational fusions integrated at the *fbp1*⁺ and *ura4*⁺ loci, respectively (Hoffman and Winston 1990). Yeast were grown and maintained using yeast extract agar (YEA) and yeast extract liquid (YEL) (Gutz *et al.* 1974). Defined medium EMM (MP Biochemicals) was supplemented with required nutrients at 75 mg/liter, except for L-leucine, which was at 150 mg/liter. Sensitivity to 5-fluoroorotic acid (5-FOA) was determined on SC solid medium containing 0.4 g/liter 5-FOA and 8% glucose as previously described (Hoffman and Winston 1990). Strains were grown at 30° unless otherwise indicated. Geldanamycin (InvivoGen) was used at 2 μg/ml, 5 μg/ml, and 10 μg/ml and was dissolved in dimethyl sulfoxide (DMSO).

Recombinant DNA methods: Rescue of plasmids from *S. pombe* was achieved by the smash and grab method (Hoffman and Winston 1987). Yeast transformations were carried out as previously described (Bähler *et al.* 1998). *Escherichia coli* transformations were done using Ten-Blue or XL1-Blue electroporation-competent cells (Stratagene, La Jolla, CA). The *S. pombe* genomic DNA insert from cosmid SPAC926 was amplified by PCR using custom oligonucleotides that divided the insert into nine segments and cloned using pNMT41 TOPO cloning vector from Invitrogen (San Diego) according to the manufacturer's instructions.

Epitope tagging of Hsp90: Oligonucleotides *hsp90*-for (5′ ATGTCGAACACAGAAACTTTCAAG 3′) and *hsp90*-revTAG (5′ ATCGACTTCCTCCATCTTGCTC 3′) were used in a PCR reaction on wild-type *S. pombe* genomic DNA to amplify the *hsp90*⁺ ORF. The resultant PCR product, lacking the *hsp90*⁺ STOP codon, was cloned into the TOPO cloning vector pNMT41 (Invitrogen), creating plasmid pMAR3, which expresses Hsp90 with a C-terminal V5 (Southern *et al.* 1991) tag followed by a hexahistidine tag (Hsp90-V5his6).

β-Galactosidase assays of *fbp1-lacZ* expression: Cells were cultured for 18 hr under repressing conditions (8% glucose) in yeast extract at the indicated temperatures (YEL). Subcultures were grown to exponential phase. Soluble protein extracts were prepared by glass bead lysis and assayed to determine β-galactosidase activity. Total soluble protein was measured by BCA assay (Pierce Chemical, Rockford, IL) to calculate β-galactosidase-specific activity (Nocero *et al.* 1994). For temperature stress experiments, cultures were pregrown as described above and then subcultured in YEL (8% glucose) such that cell density would be ~10^7 cells/ml after 6 or 24 hr incubation. Glucose concentration of the media was determined using the Sigma (St. Louis) glucose (GO) assay kit, according to manufacturer's instructions. Glucose concentrations remained >7.5% in all cultures.

TABLE 1

Strain list

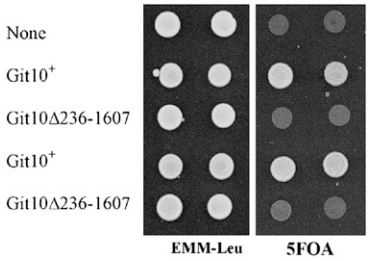
Strain	Genotype
FWP17	*mat2-102 ura4-294 lys1-131*
FWP72	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32*
FWP87	*h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32*
CHP567	*h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 git10-201*
CHP573	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366 git10-201*
CHP894	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 lys1-131 cdc1-P13 git10-201*
CHP981	*h⁻ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 swo1-26*
CHP979	*h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 ade6-M210* his7-366 *swo1-26*
CHP989	*h⁺ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 swo1-21*
PR164	*h⁻ ura4-D18 leu1-32 swo1-21*
PR165	*h⁻ ura4-D18 leu1-32 swo1-25*
CHP362	*h⁹⁰ leu1-32 ade6-M210 lys1-131*
CHP558	*h⁹⁰ fbp1::ura4⁺ leu1-32 ade6-M216 git2-1::LEU2*
CHP486	*h⁹⁰ leu1-32 lys1-131 git5-1::his7*
CHP483	*h⁹⁰ ura4::fbp1-lacZ leu1-32 ade6-M216*
MAP1	*h⁹⁰ fbp1::ura4⁺ ura4::fbp1-lacZ leu1-32 git10-201*

DNA sequencing: Mutant alleles of the *hsp90*⁺ gene (*swo1-21*, *swo1-25*, *swo1-26*, and *git10-201*) were PCR amplified from *S. pombe* strains and the PCR products were directly sequenced using custom oligonucleotides (Integrated DNA Technologies). DNA sequencing was performed using the CEQ DTCS-Quick Start kit (Beckman Coulter).

RESULTS

Genetic mapping and cloning of the *S. pombe git10*⁺ gene: Git⁻ mutant strains display 5-FOA-sensitive (5-FOA^S) growth due to their inability to glucose repress the *fbp1-ura4*⁺ reporter (Hoffman and Winston 1990). To date, nine *git* genes have been shown to play a significant role in *fbp1*⁺ repression, with only *git10*⁺ remaining to be cloned. Due to the large number of multicopy suppressors encountered when screening plasmid libraries during attempts to clone genes in this pathway (Hoffman and Winston 1991; Jin *et al.* 1995; Dal Santo *et al.* 1996; Wang *et al.* 2005), we took a genetic mapping approach to identify the *git10*⁺ gene.

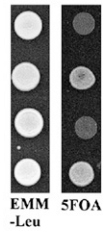
Chromosomal mapping of *git10-201* by benomyl-induced haploidization of an *h⁻/mat2-102* diploid strain (Alfa *et al.* 1993) was carried out with strains FWP17 and CHP573 (Table 1). This technique allows the formation of haploids from a diploid strain in the absence of



B

Host Genotype	Plasmid	Plasmid product	β-gal activity
git10-201	pNMT41	None	691±104
git10-201	pMAR1	Git10+	51± 17
git10-201	pMAR1A	Git10Δ*Nru*I	272± 17
git10-201	pMAR2	Git10+	41± 25
git10-201	pMAR2B	Git10Δ *Nru*I	526±146

C



Host Genotype	Plasmid	Plasmid product
git10-201	pNMT41	None
git10-201	pMAR3	Git10+V5his6
git10-201	pNMT41	None
git10-201	pMAR3	Git10+V5his6

FIGURE 1.—Complementation of *git10-201* mutation by plasmid-expressed *git10*+. (A) CHP567 (*git10-201*) cells were transformed to Leu+ with pNMT41 (empty vector), pMAR1 (*git10*+), pMAR1A (*git10Δ236-1607*), pMAR2 (*git10*+ cloned in the opposite orientation to that of pMAR1), and pMAR2B (*git10Δ236-1607* cloned in the opposite orientation to that of pMAR1A). The *git10Δ236-1607* contains a partial dropout of the *git10* ORF. The two independent transformants of each plasmid indicated were spotted on EMM −leu and then replica plated after 2 days to EMM −leu and 5-FOA plates. Plates were photographed after 3 days incubation at 30°. (B) β-Galactosidase activity was determined as described in MATERIALS AND METHODS. The values represent the average ± standard deviation of at least two independent transformants. (C) Plasmid pMAR3 carries only the *hsp90* ORF, while plasmids pMAR1 and pMAR2 carry larger segments of the chromosomal DNA that include the *hsp90*+ gene. Plasmid pMAR3 complements the *git10-201* mutation whereas pNMT41 (empty vector) does not. Transformants were spotted on EMM −leu and then replica plated after 2 days to EMM −leu and 5-FOA plates. Plates were photographed after 3 days incubation at 30°.

meiotic recombination, such that the alleles on each of the three parental chromosomes form individual linkage groups. All 5-FOA-sensitive haploids produced this way possessed chromosome 2 from CHP573, containing the *fbp1-ura4*+ reporter, as well as chromosome 1 from CHP573, presumably possessing *git10-201* (data not shown). The *git10-201* allele was further mapped by tetrad dissection, in a cross of strain FWP87 with strain CHP894. The *git10*+ gene maps between *lys1*+ (23.2 cM with a PD:TT:NPD ratio of 45:39:0) and *cdc1*+ (30.4 cM with a PD:TT:NPD ratio of 38:45:1). The *lys1*+ and *cdc1*+ genes are 54.8 cM from each other with a PD:TT:NPD ratio of 22:56:6.

The genetic mapping data suggested that *git10*+ is present on cosmid SPAC926 [one of an ordered set of cosmids used in the *S. pombe* genome sequencing project (WOOD *et al.* 2002)]. Insert DNA from SPAC926 was divided into nine fragments by PCR amplification and TOPO cloning into a plasmid suitable for transformation of *S. pombe*. Plasmids from this set of clones were used to transform *S. pombe* strain CHP567 (*git10-201*) to Leu+ and transformants were tested for restoration of 5-FOA resistance to indicate complementation of the *git10*− defect. Plasmids pMAR1 and pMAR2, which carry base pairs 2308–9026 in either orientation with respect to the vector, were the only clones to confer 5-FOA resistance (Figure 1A). These transformants also glucose-repress *fbp1-lacZ* expression as judged by β-galactosidase assays (Figure 1B). Plasmids pMAR1 and pMAR2 contain two genes, one of which is *hsp90*+/*swo1*+. Digestion with *Nru*I followed by ligation removed a 1.4-kb fragment internal to the *hsp90*+ open reading frame and produced plasmids pMAR1A and pMAR2B, which lost the ability to suppress the *git10-201* mutation (Figure 1, A and B). Thus, *hsp90*+ appears to be responsible for suppression of the *git10-201* mutant allele.

To confirm that *hsp90*+ is *git10*+, plasmid pMAR3 was constructed to express an epitope-tagged form of Hsp90 (see MATERIALS AND METHODS). CHP567 (*git10-201*) transformants carrying pMAR3 are 5-FOA resistant (Figure 1C), proving that *hsp90*+ is able to suppress the *git10-201* mutation. In contrast, transformation by pMAR3 fails to suppress the PKA pathway mutations *git1*−, *git2*− (*cyr1*−), *git7*−, or *pka1*− (data not shown). This is not surprising as Hsp90 is likely one of the most abundant proteins in *S. pombe* such that *nmt41*-driven expression of the Hsp90-V5his6 protein would not significantly increase Hsp90 activity in these strains.

Hsp90 is required for nutrient regulation of sexual development: Wild-type *S. pombe* requires either a glucose or a nitrogen starvation signal to initiate mating and meiotic entry (STETTLER *et al.* 1996). Consequently, mutations in genes required for glucose/cAMP signaling allow cells to mate and sporulate even in a nutrient-rich medium (MAEDA *et al.* 1990; ISSHIKI *et al.* 1992; JIN *et al.* 1995; LANDRY *et al.* 2000; WELTON and HOFFMAN 2000; LANDRY and HOFFMAN 2001; SCHADICK *et al.* 2002; KAO *et al.* 2006). Consistent with a role in this pathway, the *git10-201* allele of *hsp90*+ allows homothallic (h^{90}) cells to mate in a glucose-rich medium, as evidenced by presence of meiotic asci (Figure 2). This starvation-independent mating is similar to that conferred by deletion of the adenylate cyclase gene (*git2*+) or the Gβ subunit gene (*git5*+; Figure 2). Addition of 5 mM cAMP to the medium suppresses conjugation in all three mutant strains (Figure 2). This starvation-independent, cAMP-suppressible defect in the regulation of sexual development is another indication that Hsp90 plays a role in the *S. pombe* glucose/cAMP-signaling pathway.

Genetic, environmental, and chemical insults to Hsp90 activity derepress *fbp1-lacZ* expression: To investigate the role of Hsp90 in the regulation of *fbp1*+

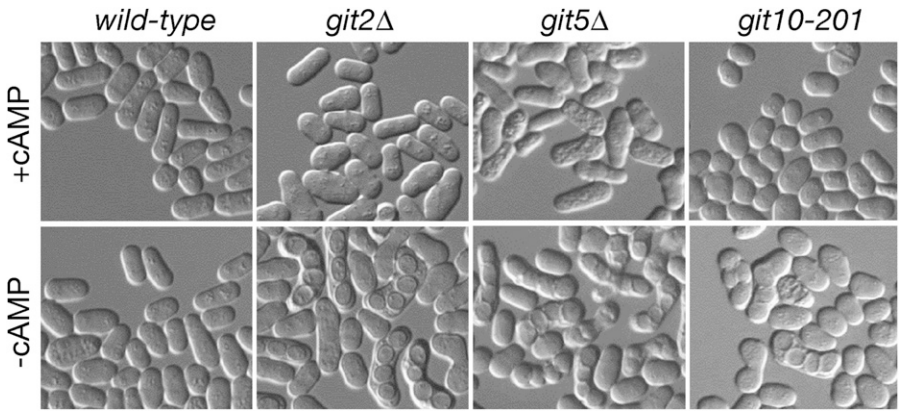


FIGURE 2.—Homothallic *git10-201* cells conjugate and sporulate in nutrient-rich medium, similar to other cAMP pathway mutants. Homothallic (h^{90}) strains CHP362 (*git10*$^+$), CHP558 (*git2*Δ), CHP486 (*git5*Δ), and MAP1 (*git10-201*) were grown to exponential phase in liquid medium (8% glucose) at 37° (to inhibit conjugation), diluted to 10^6 cells/ml in liquid medium in the presence or absence of 5 mM cAMP, and incubated overnight at 30° without shaking. Starvation-independent conjugation and sporulation, which is suppressible by addition of cAMP, is observed in all three mutant strains.

transcription, β-galactosidase activity expressed from the *fbp1-lacZ* reporter was measured in wild-type, *git10*$^-$, and *swo1*$^-$ mutant strains grown at various temperatures (Table 2). Both the *swo1-21* and the *swo1-26* alleles confer a temperature-dependent defect in *fbp1-lacZ* repression, in addition to a temperature-sensitive growth defect. The *git10-201* allele also confers a temperature-dependent defect in *fbp1-lacZ* repression; however, these cells remain viable when cultured at 37°. Surprisingly, wild-type cells display a partial defect in *fbp1-lacZ* repression when cultured at 37°, suggesting that temperature stress of wild-type cells leads to a reduction in PKA activity, and not simply the activation of the Spc1/Sty1 MAPK required for *fbp1*$^+$ transcription (see DISCUSSION).

The effect of temperature stress on *fbp1-lacZ* repression was further examined in a time-course experiment in which wild-type cells were cultured at 30° or 40°, a temperature that does not support growth of *S. pombe*, but at which cells remain viable for several days (C. A. HOFFMAN and C. S. HOFFMAN, unpublished results). Increased β-galactosidase activity in response to temperature stress can be detected within 1 hr (data not shown) and remains modest even after 6 hr of incubation (Figure 3A). By 24 hr, however, the β-galactosidase activity rises to 547 ± 80 units, demonstrating that prolonged exposure to heat stress is required for significant *fbp1*$^+$ derepression. As the glucose levels in the media remain >7.5% in all cultures, the increased *fbp1-lacZ* expression is due to heat stress and not glucose starvation.

To independently test whether Hsp90 is required for *fbp1*$^+$ regulation, we examined the effect of chemical inhibition of Hsp90 on *fbp1-lacZ* expression by exposing cells to the Hsp90 inhibitor geldanamycin (WHITESELL *et al.* 1994). β-Galactosidase activity was measured from wild-type strain FWP77 cells grown at 30° for 18 hr in the presence or the absence of geldanamycin (2 μg/ml, 5 μg/ml, and 10 μg/ml). There was a clear dose-dependent derepression of *fbp1-lacZ* expression, although the levels of expression did not reach those detected in cells subjected to prolonged heat stress (Figure 3B).

Phenotypic differences between *swo1*$^-$ and *git10*$^-$ alleles of *hsp90*$^+$: In the course of assaying β-galactosidase activity from *swo1*$^-$ and *git10*$^-$ strains, we confirmed previous observations that indicated that the *swo1*$^-$ alleles confer temperature-sensitive growth (ALIGUE *et al.* 1994), while the *git10-201* allele does not (HOFFMAN and WINSTON 1990). For a more rigorous comparison, we carried out spot tests on *hsp90*$^+$, *swo1-26*, *swo1-21*, and *git10-201* strains to examine growth on rich medium at 25°, 28°, 30°, and 37°. Both *swo1*$^-$ mutants display a severe temperature-sensitive growth defect, even at 30°, while the *git10-201* mutants display only a slow-growth phenotype at 37° rather than a loss of cell viability (Figure 4).

TABLE 2

Glucose repression of *fbp1-lacZ* expression as a function of growth temperature

		β-Galactosidase activity				
Strain	*hsp90* allele	25°	27°	30°	32°	37°
FWP87	Wild type	15 ± 5	11 ± 0	10 ± 6	12 ± 4	392 ± 6
CHP567	*git10-201*	154 ± 20	252 ± 26	626 ± 30	661 ± 157	1336 ± 131
CHP981	*swo1-26*	54 ± 3	144 ± 5	517 ± 55	Inviable	Inviable
CHP989	*swo1-21*	157 ± 12	377 ± 8	605 ± 105	Inviable	Inviable

β-Galactosidase activity was measured in cells growing in YEL medium under glucose-repressing conditions (8% glucose) for 18 hr at the indicated temperature. The values given represent specific activity average ± standard deviation from two or three independent cultures.

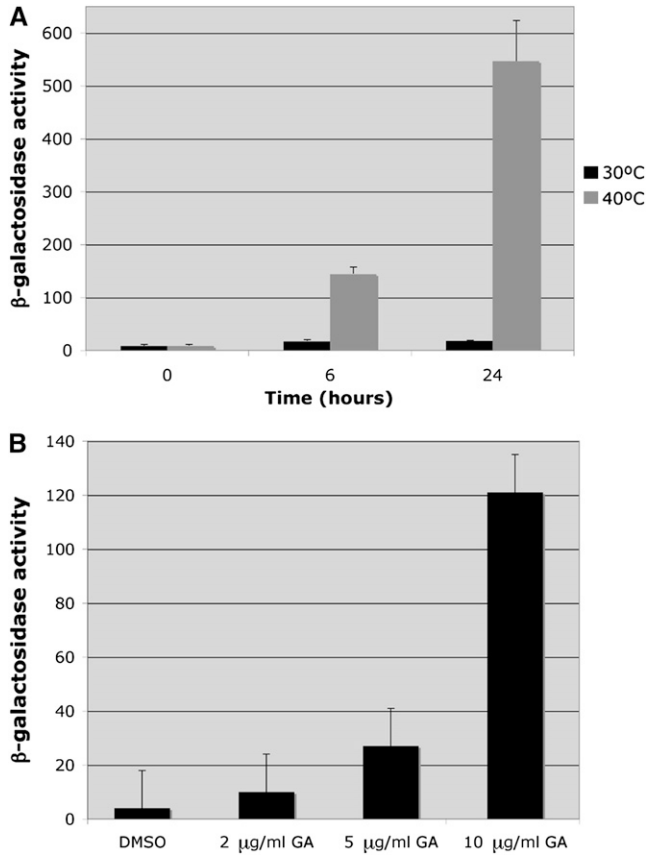


FIGURE 3.—Prolonged heat stress and chemical inhibition of Hsp90 derepress *fbp1-lacZ* transcription. (A) Wild-type strain FWP77 was pregrown to exponential phase at 30° and then subcultured at 30° or 40° in YEL medium under glucose-repressing conditions. β-Galactosidase activity was measured at the times indicated. The values given represent specific activity average ± standard deviation from three independent cultures. Glucose levels in the media were ≥7.5% for each culture. (B) β-Galactosidase activity was measured in cells growing in 8% glucose YEL medium for 18 hr in the presence of the Hsp90 inhibitor geldanamycin at the indicated concentrations. The values given represent specific activity average ± standard deviation from two or three independent samples.

Microscopic examination of *hsp90*$^+$, *swo1*$^-$, and *git10-201* strains growing at 28°, 30°, and 37° was carried out to examine the nature of the temperature-dependent growth defect. After 24 hr growth on EMM defined medium, the *swo1-21* strain displayed abnormal cells that were lysed or binucleate or with misplaced nuclei in cultures grown at 30° and 37° (Figure 5). The *swo1-26* strain appeared normal at 30°, while most cells had improperly placed nuclei at 37°. These results contrast somewhat with those from the spot test of a *swo1-26* strain at 30° (Figure 4), and this appears to be a medium-specific effect with these cells displaying a more severe growth defect on YEA rich medium than on EMM defined medium. No growth defects were observed in wild-type or *git10-201* cells at any temperature (Figure 6), distinguishing the cAMP pathway defect caused by the *git10-201* mutation from the cell-growth defects caused by the *swo1-21* and *swo1-26* mutations.

Sequence analysis of *swo1*$^-$ and *git10*$^-$ alleles: The sequence of the entire *hsp90*$^+$ open reading frame was determined from strains carrying the *swo1-21*, *swo1-25*, *swo1-26*, and *git10-201* alleles. The *swo1-25* and *swo1-26* alleles carry the same mutation, changing residue 84 from glycine to cysteine, while the mutation in *swo1-21* changes residue 654 from leucine to arginine. The *git10-201* allele changes residue 338 from leucine to proline (Figure 6A). Thus, the *swo1-25* and *swo1-26* alleles affect the N-terminal ATP-binding domain, the *swo1-21* allele affects the C-terminal dimerization domain, and the *git10-201* allele affects the central, client protein-binding domain. The locations of these mutations are consistent with the observations that the *swo1*$^-$ mutant alleles appear to be general reduction-of-function alleles, while the *git10-201* mutation appears to confer only a modest growth defect, but a significant defect in glucose/cAMP regulation of *fbp1*$^+$ transcription. A similar separation-of-function allele of an Hsp90 gene has been observed in the cGMP signaling pathway of the nematode *Caenorhabditis elegans*. The *daf-21* mutation, which allows *C. elegans* to enter the dauer larval form in the absence of temperature or nutritional stress signals (BIRNBY *et al.* 2000), is a missense mutation that alters a residue in the Hsp90 central domain not far from the residue altered by the *S. pombe git10-201* mutation (Figure 6B). The similarity between these two mutations and their associated

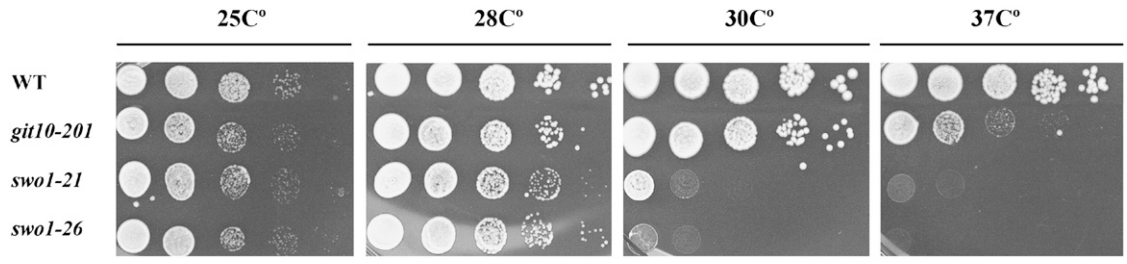


FIGURE 4.—Temperature-dependent growth of *hsp90*$^+$, *swo1-26*, *swo1-21*, and *git10-201* strains. Spot tests were carried out on YEA rich medium at 25°, 28°, 30°, and 37°. Strains FWP72 (wild type), CHP567 (*git10-201*), CHP989 (*swo1-21*), and CHP979 (*swo1-26*) were cultured to 1×10^7 cells/ml in YEL liquid medium. Cells were washed with YEL medium and adjusted to 2×10^7 cells/ml and subjected to five 10-fold serial dilutions. Five microliters of each culture were spotted to a YEA plate and grown for 3 days at the indicated temperature before photographing.

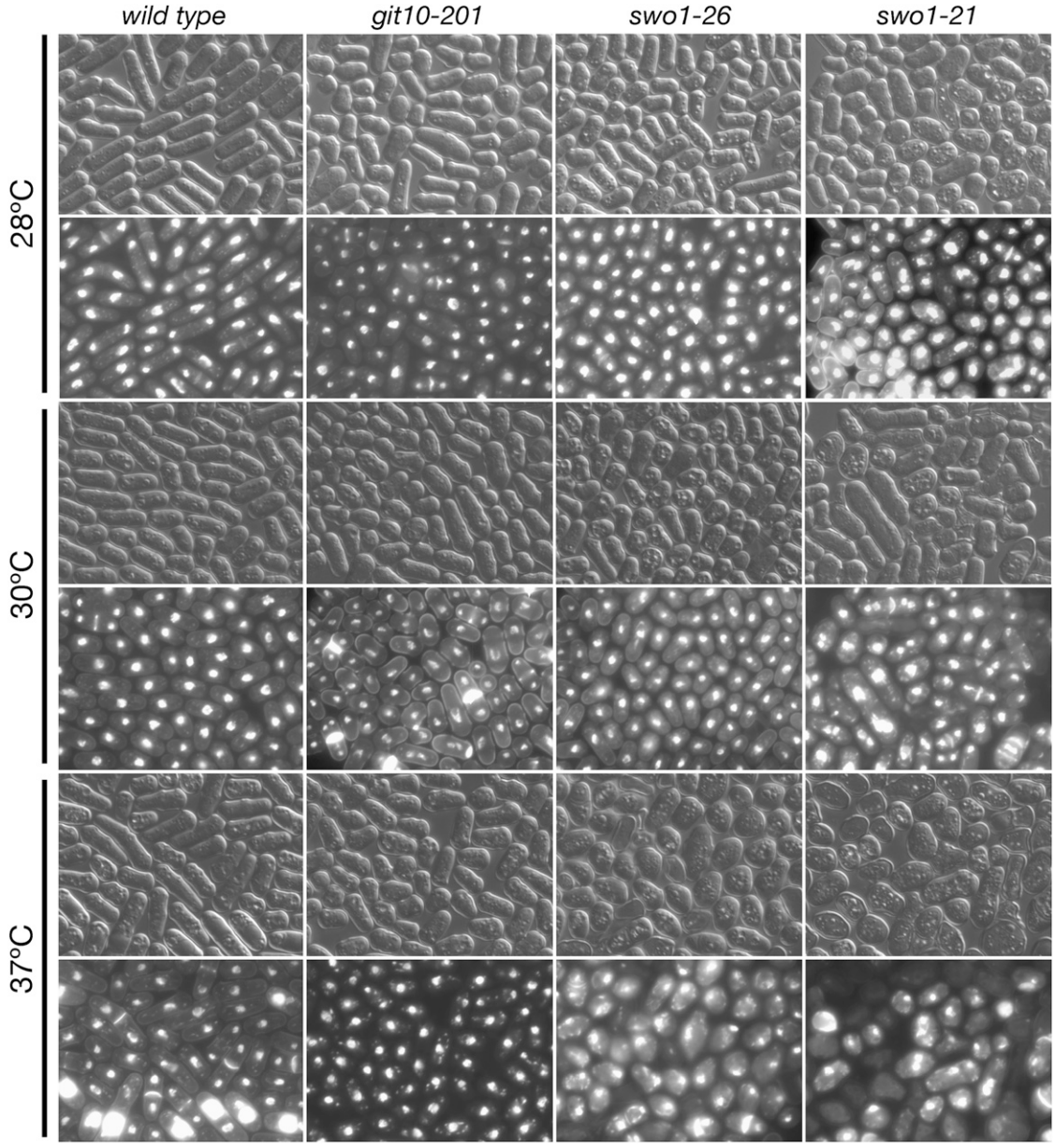


Figure 5.—Temperature-dependent morphology of *hsp90*$^+$, *swo1-26*, *swo1-21*, and *git10-201* strains. The same strains as shown in Figure 4 were precultured at 28° and then transferred to EMM defined medium and grown for 24 hr at 28°, 30°, and 37°. Cells were heat-fixed and stained with Hoechst 33342 and Calcofluor. Images were visualized and captured using a Zeiss Axioplan2 microscope with an Orca-ER CCD camera and Openlab software.

phenotypes suggests that Hsp90 plays a similar role in both *S. pombe* and *C. elegans* cyclic nucleotide signaling pathways to regulate metabolic pathways in response to temperature and nutritional conditions.

DISCUSSION

We have shown here that Hsp90, encoded by the *hsp90*$^+$/*git10*$^+$/*swo1*$^+$ gene, acts in the *S. pombe* cAMP-signaling pathway that senses environmental glucose to repress transcription of genes involved in sexual development and gluconeogenesis, such as the *fbp1*$^+$ gene (Hoffman 2005a,b). A defect in cAMP signaling in a *git10-201* mutant strain was previously demonstrated (Byrne and Hoffman 1993), as was suppression of the *fbp1*$^+$ regulatory defect by cAMP addition to the growth medium or by overexpression of the *git2*$^+$/*cyr1*$^+$ adenylate cyclase gene (Hoffman and Winston 1991). Therefore, Hsp90 activity appears to be required for cells to detect glucose and activate adenylate cyclase.

The *hsp90*$^+$ gene is one of seven required for adenylate cyclase activation, which form at least two functionally distinct groups as determined by the ability of mutations to be suppressed by the mutationally activated Gpa2^{R176H}

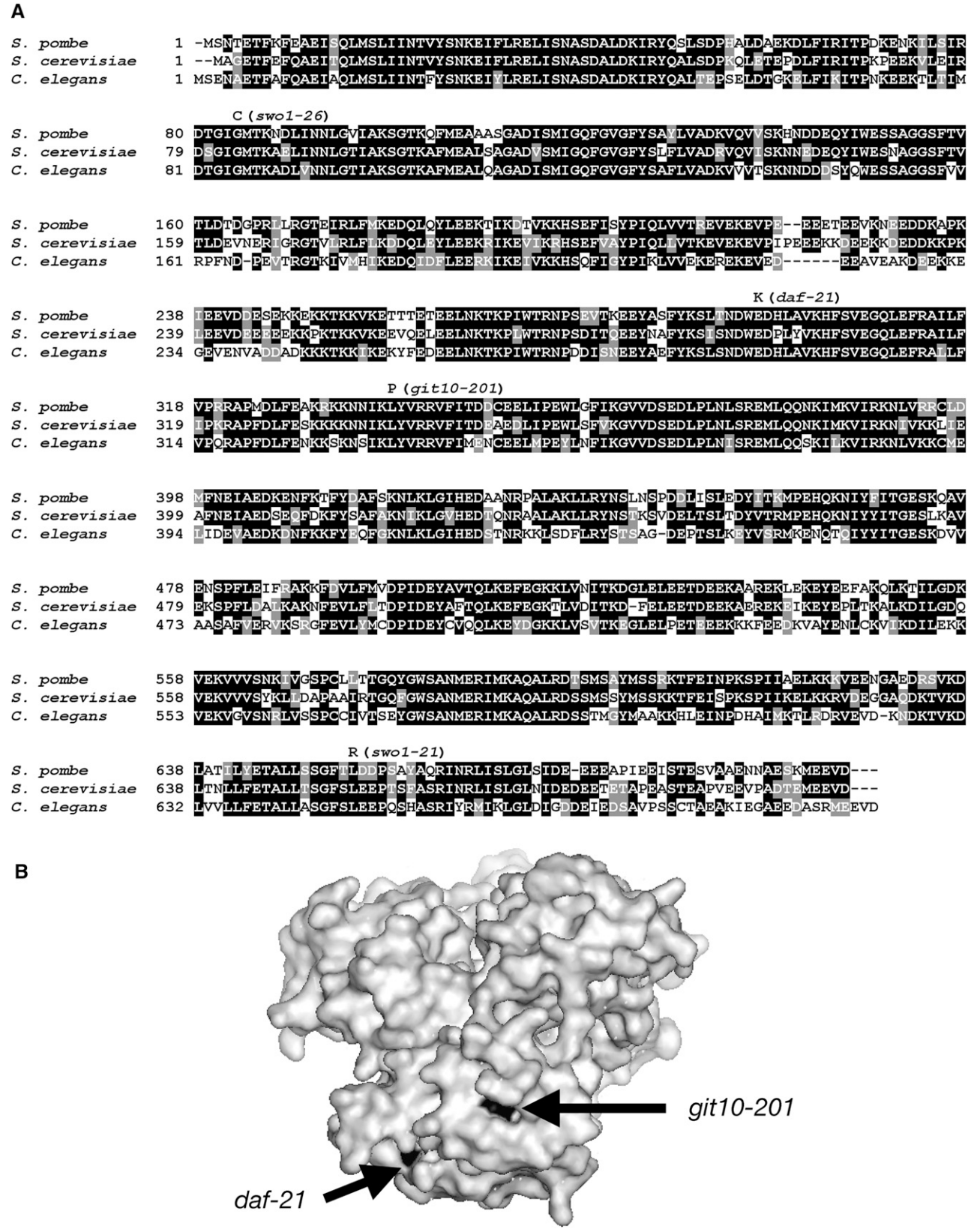


FIGURE 6.—Alignment of Hsp90 proteins from *S. pombe*, *S. cerevisiae*, and *C. elegans*. (A) The *S. pombe* Hsp90 protein (accession no. CAB54152) was aligned using ClustalW (THOMPSON *et al.* 1994) with the *S. cerevisiae* Hsc82 protein (accession no. CAA89919) and *C. elegans* DAF-21 (accession no. NP_506626) and displayed using BOXSHADE 3.21. Identical residues are shaded in black, while conserved residues are shaded in gray. Amino acid changes associated with the *swo1-21*, *swo1-26*, and *git10-201* mutant alleles are also indicated, as well as that of the *C. elegans daf-21* mutation. (B) Crystal structure of the central domain of *S. cerevisiae* Hsp82 (accession no. AAA02813) showing the location of the residues altered by the *S. pombe git10-201* mutation and the *C. elegans daf-21* mutation. The two altered residues are on the same surface of the Hsp90 central domain. The graphic image was created using Pymol (DELANO 2002).

Gα or by overexpression of the wild-type Gpa2$^+$ protein (Welton and Hoffman 2000; Landry and Hoffman 2001). Increasing Gpa2 function bypasses the loss of the Git3p GPCR or Git5-Git11 Gβγ, but not mutations affecting the Git1 C2-domain protein (Kao *et al.* 2006), the Git7 Sgt1-family member protein (Schadick *et al.* 2002), or the Git10 Hsp90 protein. While *S. cerevisiae* does not encode a Git1 homolog, the *S. cerevisiae* Sgt1 plays an undetermined role in cAMP signaling (Dubacq *et al.* 2002) and functions in kinetochore assembly as an Hsp90 cochaperone or client-adaptor protein (Bansal *et al.* 2004; Lingelbach and Kaplan 2004; Catlett and Kaplan 2006). On the basis of our finding that Hsp90 acts in the *S. pombe* cAMP pathway, we presume that it is functioning together with Git7. Notably, the *git7-27* and *git7-235* mutant alleles confer both cell-growth and cAMP-signaling phenotypes similar to those of the *swo1*$^-$ mutant alleles, while *git7-93* and a *git7-GFP* fusion allele are separation-of-function alleles similar to *git10-201* as they affect cAMP signaling, but not cell growth and division. Together, the presence of such alleles for both *git7*$^+$ and *hsp90*$^+$ suggests that these proteins act on a distinct client protein or on the assembly of a protein complex acting in the cAMP pathway and argues against a model in which mutations that reduce Git7 and/or Hsp90 activity simply create a general stress that mimics a glucose-starvation signal.

Hsp90 is an essential molecular chaperone, which is highly conserved from bacteria to mammals (Bardwell and Craig 1987; Lindquist and Craig 1988; Spence and Georgopoulos 1989). Hsp90 possesses three domains, an N-terminal ATP-binding domain, a central regulatory domain involved in client protein binding, and a C-terminal dimerization domain (Pearl and Prodromou 2006). It is an unusual chaperone in that most of its identified substrates are signal-transduction proteins (Pearl and Prodromou 2000; Zhang and Burrows 2004; Powers and Workman 2006). Precisely how the Hsp90 machinery regulates signaling pathways in cells is not fully understood, as it appears to stabilize some client proteins and function in the assembly of protein complexes for other clients.

In *S. pombe*, the Hsp90 gene was first identified as *swo1*$^+$, mutations in which suppress the mitotic effect of overexpression of the Wee1 kinase, which negatively regulates mitotic entry (Aligue *et al.* 1994). More recently, Hsp90 has been shown to play a role in myosin II function and the assembly of the *S. pombe* actomyosin ring involved in cytokinesis (Mishra *et al.* 2005). Hsp90 may be required in the glucose/cAMP pathway for the assembly of a protein complex required for signaling or may be involved in either the folding or the localization of a component of the signaling pathway. For example, Hsp90 is required together with Sgt1 in *S. cerevisiae* kinetochore assembly (Bansal *et al.* 2004; Lingelbach and Kaplan 2004), while in COS cells Hsp90 helps to localize $G\alpha_{12}$ to lipid rafts (Waheed and Jones 2002). The failure of an activated allele of the *gpa2* Gα gene to suppress the *git10-201* mutation would be consistent with either of these mechanisms of action.

The central domain of Hsp90 appears to be a major site for client protein interactions (Sato *et al.* 2000; Fontana *et al.* 2002; Meyer *et al.* 2003). A recent report showed that this domain could also play a role in distinguishing between different types of client proteins (Hawle *et al.* 2006). Therefore, the *git10-201* L338P mutation in the central domain might result in impairment of a client protein activity in the cAMP pathway specifically, whereas the temperature-sensitive mutants might result in a universal impairment of Hsp90 client proteins in the cell or contribute to general instability of Hsp90 itself. Thus, we have identified a separation-of-function allele of Hsp90, which confers a defect in cAMP signaling, but not other essential processes. A similar observation was reported in *C. elegans*; loss of Hsp90/DAF-21 involved in cGMP signaling confers an early larval lethality; however, a missense mutation affecting a residue in the middle domain produces a viable adult with a chemosensory defect (Birnby *et al.* 2000). Mapping of the residues altered by the *daf21* mutation and by the *git10-201* mutation onto the crystal structure of the *S. cerevisiae* Hsc82p central domain reveals that these two residues are in close proximity to each other (Figure 6B). Therefore, these two separation-of-function alleles may affect their individual cyclic nucleotide signaling pathways via the same mechanism.

These studies also revealed a new insight into heat stress in *S. pombe*. It has been long known that heat stress activates the Spc1/Sty1 SAPK pathway required for *fbp1*$^+$ transcription, presumably by regulating the activity of the Pyp1 tyrosine phosphatase (Samejima *et al.* 1997). Our data further indicate that in addition to activating the SAPK pathway, heat stress reduces PKA activity. Stresses such as nitrogen starvation and osmotic stress, which activate the SAPK pathway, do not derepress *fbp1*$^+$ transcription (Devoti *et al.* 1991; Stettler *et al.* 1996; Janoo *et al.* 2001; Yang *et al.* 2003; Stiefel *et al.* 2004), indicating that reduction of PKA activity is required for *fbp1*$^+$ derepression. Therefore, we have discovered a novel link between glucose and heat sensing that appears to involve Hsp90. We speculate that heat stress redirects Hsp90 from acting in the cAMP pathway to acting upon targets that are critical to survival of heat stress. As a secondary effect, the ability of heat stress to reduce PKA activity and thus mimic glucose starvation may assist in producing a growth arrest that enhances cell survival at elevated temperatures.

In summary, we have shown that attenuating Hsp90 function by mutation, pharmacological inhibition, or temperature stress impairs cAMP-mediated glucose signaling, consistent with a specific role for Hsp90 in the glucose/cAMP pathway. Further studies will be directed to investigating the mechanism by which Hsp90 acts in this partcular pathway. Given the evidence that the

Hsp90 cochaperone Git7/Sgt1 acts in both *S. pombe* and *S. cerevisiae* cAMP pathways, it is likely that Hsp90 will be found to function in the *S. cerevisiae* cAMP pathway as well as in cAMP pathways of other yeasts and fungi.

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