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A *trans*-Activation Domain in Yeast Heat Shock Transcription Factor Is Essential for Cell Cycle Progression during Stress

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Gene expression in response to heat shock is mediated by the heat shock transcription factor (HSF), which in yeast harbors both amino- and carboxyl-terminal transcriptional activation domains. Yeast cells bearing a truncated form of HSF in which the carboxyl-terminal transcriptional activation domain has been deleted [HSF(1-583)] are temperature sensitive for growth at 37°C, demonstrating a requirement for this domain for sustained viability during thermal stress. Here we demonstrate that HSF(1-583) cells undergo reversible cell cycle arrest at 37°C in the G_2/M phase of the cell cycle and exhibit marked reduction in levels of the molecular chaperone Hsp90. As in higher eukaryotes, yeast possesses two nearly identical isoforms of Hsp90: one constitutively expressed and one highly heat inducible. When expressed at physiological levels in HSF(1-583) cells, the inducible Hsp90 isoform encoded by *HSP82* more efficiently suppressed the temperature sensitivity of this strain than the constitutively expressed gene *HSC82*, suggesting that different functional roles may exist for these chaperones. Consistent with a defect in Hsp90 production, HSF(1-583) cells also exhibited hypersensitivity to the Hsp90-binding ansamycin antibiotic geldanamycin. Depletion of Hsp90 from yeast cells wild type for HSF results in cell cycle arrest in both G_1/S and G_2/M phases, suggesting a complex requirement for chaperone function in mitotic division during stress.

In response to thermal stress, eukaryotic cells mount a protective and adaptive response by activating the expression of heat shock genes encoding a family of highly conserved proteins. Many heat shock proteins have been shown to function as molecular chaperones, playing roles in folding or unfolding of substrates, protein trafficking and degradation, and maintenance of protein conformation. The Hsp90 family is conserved from bacteria to humans, and its members have been shown to function as molecular chaperones in vitro (51, 52, 70) and in vivo (44). *Saccharomyces cerevisiae* possesses two genes encoding highly similar isoforms of Hsp90, the constitutively expressed *HSC82* gene and a highly heat-inducible gene, *HSP82* (8). The expression of at least one of these genes is essential for viability even under normal growth conditions, and Hsp90 is required at elevated levels during thermal stress (8), demonstrating that Hsp90 is required in both stressed and unstressed cells. One of the best-studied roles of Hsp90 is that as a chaperone for mammalian steroid hormone receptors (47), which when expressed in yeast require Hsp90 for stability and maintenance of the inactive state as well as maximal transcriptional activity in the presence of ligand (43, 46). Receptor-Hsp90 complexes purified from higher eukaryotes also contain a number of other associated proteins, many of which possess chaperone activity themselves (28, 56). Similar complexes have been identified in yeast (11) and include an Hsp70 isoform, the yeast DnaJ chaperone homolog Ydj1p (32), Sti1p (12), the yeast cyclophilin homologs Cpr6p and Cpr7p (17), and Cdc37p (31, 62). Hsp90 has been shown to interact with a number of important mammalian proteins involved in signaling and growth control such as the glucocorticoid receptor (GR) and $pp60^{v\text{-}src}$ (69). The roles of this chaperone in *S. cerevisiae*, however, remain elusive.

In yeast, two overlapping but distinct systems represented by the Msn2p and Msn4p transcription factors and the heat shock transcription factor (HSF) coordinate the induction of genes encoding heat shock proteins in response to heat stress (39, 49). HSF binds to specific *cis*-acting heat shock elements composed of repeating nGAAn blocks as a trimer via its DNA binding domain, which lies adjacent to a hydrophobic oligomerization region (7, 41, 58, 68). Whereas mammalian, fly, and plant HSFs harbor a single *trans*-activation domain, two separate transcriptional activation domains have been characterized in the *S. cerevisiae* HSF: an amino-terminal transcriptional activation domain (residues 1 to 172) and a carboxyl-terminal transcriptional activation domain (CTA; residues 584 to 833) (45, 57). Although the HSF CTA is dispensable at temperatures of approximately 34°C or less, it is required for growth at heat shock temperatures (13, 45, 55). The CTA shows little sequence conservation with other HSF proteins but possesses potent transcriptional activation potential (13). Moreover, expression of the *SSA1*, *SSA3*, and *SSA4* genes, encoding yeast Hsp70 isoforms, is only modestly reduced in cells carrying an HSF protein that is truncated at residue 583 [HSF(1-583)] and therefore lacks the CTA, suggesting that the two transcriptional activation domains may be differentially utilized for the control of distinct target genes (57, 64, 71). This model is supported by the observation that the HSF CTA is required for expression of the yeast metallothionein gene *CUP1* in response to heat, oxidative stress, and glucose starvation (35, 64). The apparent temperature-sensitive growth phenotype of the HSF(1-583) strain (57) suggests that the expression of one or more specific gene products under CTA control is required for growth at high temperature.

Here we demonstrate that cells expressing the HSF derivative HSF(1-583) lacking the CTA undergo reversible cell cycle arrest at 37°C in the G_2/M phase of the *S. cerevisiae* cell cycle. A genetic selection for multicopy suppressors of this phenotype revealed that the heat-inducible yeast Hsp90 isoform *HSP82* was capable of allowing cell cycle progression at elevated temperatures in this strain. Suppression was dependent on Hsp90 activity, as a temperature-sensitive allele of *HSP82*

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was unable to complement loss of the HSF CTA. HSF(1-583) cells were found to be severely deficient in expression of both Hsp90 genes as judged by immunoblot analysis, and depletion of Hsp90 from wild-type yeast cells partially recapitulated the cell cycle arrest phenotype, demonstrating an essential role for the HSF CTA and Hsp90 in cell cycle progression during thermal stress.

MATERIALS AND METHODS

Strains and plasmids. Two different sets of isogenic wild-type and HSF(1-583) strains were used in this study. Strains DTY123 (*MAT***a** *his4-539 ura3-52 lys2-801 SUC2*) (10) and DTY179 [*MAT***a** *his4-539 ura3-52 lys2-801 SUC2 HSF(1-583)::URA3*] were used for characterization of the cell cycle arrest phenotype. The HSF(1-583) truncation mutation was previously described and includes two tandem stop codons which effectively prevent translational readthrough (64), as previously reported for other *HSF1* nonsense mutations (34). Due to the paucity of auxotrophic markers in this strain background, further experiments were carried out with variants of the strain PS145 (*MAT***a** *ade2-1 trp1 can1-100 leu2-3-112 his3-11,-15 ura3 hsf1::LEU2 [pGAL-HSF1::URA3]*), which carries a plasmid-borne copy of the *HSF1* gene under the control of the galactose-inducible *GAL1* promoter (59). To obtain strains which did not require galactose as a carbon source for *HSF1* expression, plasmids pRS314HSF and pRS314HSF(1-583) were independently transformed into PS145. Trp^+ transformants were replated on 5-fluoroorotic acid to select for colonies which had spontaneously lost the pGAL-HSF1::URA3 plasmid carrying the wild-type *HSF1* allele (5). The resultant strains PS145* and PS145*HSF(1-583) are referred to in the text as HSF and HSF(1-583), respectively, for clarity and display indistinguishable growth parameters compared to strains DTY123 and DTY179, respectively. Strain 5CG2 (*MAT*α *ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 hsc82::URA3 hsp82::GAL1-HSP82::LEU2*) (a kind gift of S. Lindquist, University of Chicago) was used for depletion of Hsp90 isoforms in a wild-type HSF genetic background (30, 46).

Plasmid pYEp24*HSC82* was constructed by subcloning a 5.1-kb *Bam*HI fragment encompassing the entire *HSC82* gene and flanking sequences from plasmid pUTX203 (S. Lindquist) into pYEp24 linearized with *Bam*HI (8). To place *HSP82* genes under transcriptional control of the glyceraldehyde phosphate dehydrogenase (GPD) promoter on a *HIS3*-based expression plasmid, 3.1-kb *Bam*HI fragments including either the wild-type *HSP82* open reading frame or the temperature-conditional $HSP82^{G170D}$ allele were subcloned from plasmids pHGPD and pTGPD-G170D (S. Lindquist), respectively, into pRS413GPD (42, 43). Plasmid p413GPD*HSC82* was constructed by amplifying the *HSC82* coding region only with the following primers. Primer C82BAMUP (CGCTACgGAtC CAATAGAAAAATAG) hybridized to the 5′ untranslated region (−40 to −15, relative to ATG). Primer C82XHODN (CCAACTTTTTTAAAGGCGCcTcgA GCAG) hybridized to the 3′ untranslated region of the gene (+2191 to +2219). Lowercase letters in the DNA sequence represent mutations in the sequence which were introduced to create *Bam*HI and *Xho*I sites (underlined), respectively. A 2.2-kb PCR product was obtained from plasmid pUTX203 by using *Pwo* high-fidelity polymerase (Boehringer Mannheim) and subcloned into plasmid p413GPD. Plasmid p413GPDGR, expressing a rat cDNA encoding the GR (a kind gift of D. Robins, University of Michigan, Ann Arbor), will be described elsewhere. Plasmid pYRP-GRElacZ was a kind gift of D. McDonnell, Duke University.

High-copy suppressor screen. Strain PS145*HSF(1-583) was transformed with a high-copy yeast genomic DNA library in the *URA3*-based YEp24 vector (9) and plated onto synthetic complete (SC)-uracil plates. After overnight incubation at 30°C, which allowed recovery from the transformation but only limited growth, plates were shifted to 37°C and incubated for 3 days and suppressor colonies were identified. From approximately 60,000 independent transformants screened, six plasmids which conferred growth on fresh PS145*HSF(1-583) cells when retransformed were recovered. These six were placed into classes based on restriction digestion patterns, and representative clones were sequenced from both ends of the insert by using standard T3 and T7 primers to identify the genomic fragments present.

Growth conditions and microscopy. Rich yeast medium (YPD) was used for growth of DTY123 and DTY179; all other strains bearing plasmids were propagated in SC medium lacking the indicated nutrients. Strain 5CG2 was grown in rich medium with 2% galactose as the sole carbon source (YPGal) unless otherwise indicated. Growth at various temperatures was assessed by serially diluting cultures from an initial concentration of ca. 2×10^6 cells/ml by factors of 10 in 150-μl volumes in a sterile 96-well plate. A 48-pin multipronged replicator was used to transfer approximately 3 μl of cell suspensions to SC plates lacking the appropriate nutrients to maintain plasmids.

To prepare cells for microscopy, a 5-μl culture aliquot was mixed with an equal volume of warm 1% low-melting-point agarose on a glass slide and immediately sealed beneath a coverslip. Slides were viewed with differential interference contrast (Nomarski) optics or fluorescent illumination through the appropriate filter for diamidinophenylindole (DAPI) staining, with a Zeiss Axioskop microscope (Oberkochen, Germany). Photomicrographs were obtained by using a Zeiss MC80 microscope camera and Kodak Ektaprint 400 slide film. Slides were digitally scanned and composed with Adobe Photoshop software (Adobe Systems, Inc., Mountain View, Calif.).

Flow cytometry. A revised protocol based on a published procedure (29) was followed to prepare cells for flow cytometric analysis of DNA content. At the indicated time points, approximately 10^7 cells were harvested by centrifugation, resuspended in 70% ethanol, and incubated for 30 min at room temperature with agitation. Cells were then washed three times with 50 mM sodium citrate (pH 7.0), resuspended in the same buffer containing 40 μg of RNase A per ml, and incubated at 37°C for 2 h. The RNase A was removed by two washes in the sodium citrate buffer, and cells were resuspended in staining buffer (100 μg of propidium iodide per ml, 50 mM sodium citrate [pH 7.0], 10 mM NaCl, 0.1% Nonidet P-40) and incubated for 30 min at room temperature. Following staining, cells were washed three times with storage buffer (10 μg of propidium iodide per ml, 50 mM sodium citrate, 10 mM NaCl, 0.1% Nonidet P-40), and stored at 4°C in the same buffer until analysis. Analysis of DNA content was performed with a Coulter Epics flow cytometer (Coulter Corporation, Miami, Fla.) on at least 10,000 cells per sample.

Protein extraction and immunoblotting. Whole-cell protein extracts for immunoblotting were prepared by glass bead extraction in MURB buffer (50 mM sodium phosphate–25 mM morpholineethanesulfonic acid [MES] [pH 7.0], 3 M urea, 1% sodium dodecyl sulfate, 0.5% 2-mercaptoethanol, 1 mM sodium azide) (23) with the Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, Ind.) for 5 min at room temperature with a multimixer apparatus (model MT-360; Tomy, Palo Alto, Calif.). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, Calif.). Extracts were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with standard conditions, and proteins of interest were detected with the Renaissance chemiluminescence detection system (NEN Life Sciences, Boston, Mass.). Antiserum against Hsc/p82p was a kind gift of S. Lindquist, and a purified monoclonal antibody against yeast phosphoglycerate kinase was obtained from Molecular Probes (Eugene, Oreg.). Band intensity was estimated by using NIH Image software (v1.61; National Institutes of Health) and by averaging results obtained from multiple exposures.

Geldanamycin inhibition of GR function. Wild-type and HSF(1-583) cells transformed with pYRP-GRElacZ and either pRS413GPD (no GR control) or pRS413GPDGR were grown in selective minimal medium at 30°C to a density of approximately 10^7 cells/ml, harvested, and resuspended to 10^8 cells/ml. Four equal 1-ml aliquots of pRS413GPDGR-containing cells were treated with appropriate dilutions of a 5 mM stock concentration of geldanamycin (obtained from W. Pratt, University of Michigan) in dimethyl sulfoxide (DMSO) to achieve the indicated final concentrations and incubated with shaking for 30 min. Geldanamycin-treated cells were then further divided into equal 0.5-ml aliquots, to which 0.5 ml of fresh medium containing either 2× (20 μM) deoxycorticosterone (DOC; Sigma) or the vehicle ethanol alone was added, followed by shaking at 30°C for another 60 min. The experiment was terminated by transferring the cells to ice, and cell pellets obtained after centrifugation were flash-frozen on dry ice. Total cellular RNA preparation, RNase protection assay, and data quantitation were as previously described (35).

Geldanamycin toxicity assay. To determine sensitivity to geldanamycin, cultures were grown overnight and diluted to ca. 2×10^6 cells/ml. Two hundred microliters of diluted culture was then mixed with 3 ml of SC top agar (0.8% agar) lacking uracil and spread evenly over a standard prewarmed SC-uracil plate. After cooling and solidification, 10-μl aliquots of geldanamycin (2 mM in DMSO) or DMSO alone were spotted onto the top agar and allowed to dry. Plates were then incubated for 2 days and photographed directly or under low-power (×25) magnification.

RESULTS

HSF(1-583) is temperature sensitive for growth due to cell cycle arrest at 37°C. Two mutant alleles of *HSF1* which lack the CTA [HSF(1-424) and HSF(1-583)] were previously demonstrated to display no obvious growth defects at 30°C but fail to grow at temperatures of 37°C or greater (Fig. 1A) (57). Although HSF(1-583) is competent for heat induction of the yeast Hsp70 genes *SSA1*, *SSA3*, and *SSA4* (64, 71), the *CUP1* gene exhibits a strong requirement for the HSF CTA for transcription in response to heat, glucose starvation, and oxidative stress (35, 64). Furthermore, removal of the CTA does not result in degradation or aberrant oligomerization of HSF: the HSF(1-583) protein is as stable as wild-type HSF and is maintained as a trimer during prolonged incubation at 37°C (data not shown). Upon microscopic examination, we observed that HSF(1-583) cells shifted to heat shock temperatures were morphologically distinct from wild-type control cells subjected to the same treatment. To more thoroughly investigate this phe-

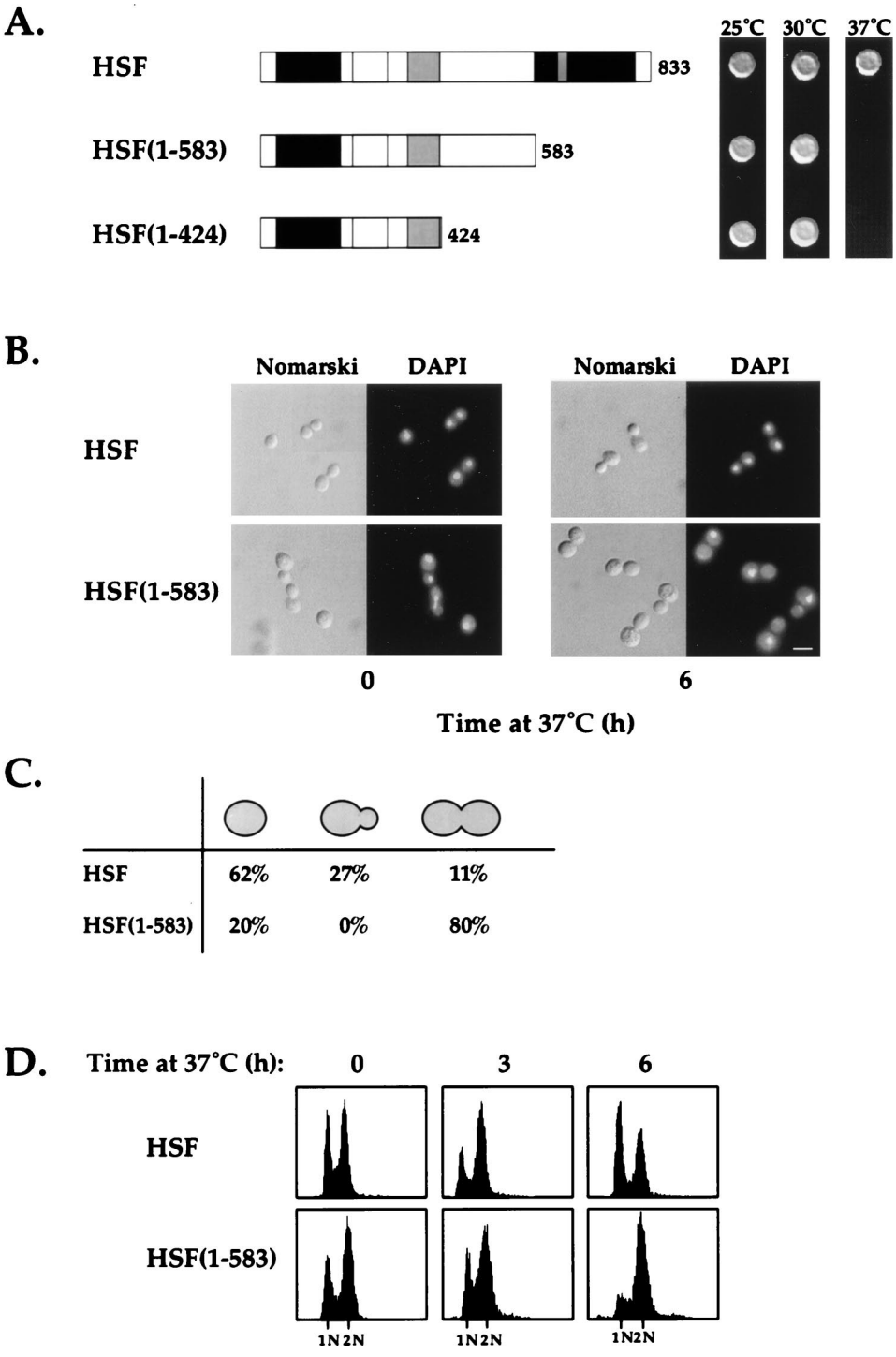

FIG. 1. HSF(1-583) cells undergo cell cycle arrest at the nonpermissive temperature. (A) Wild-type and truncated HSF molecules are depicted schematically along with their respective growth phenotypes at the indicated temperatures. The numbers above each HSF derivative indicate the last residue in the truncated molecule numbered with respect to the wild-type protein. The domains of HSF are designated as follows: black, transcriptional activation; light grey, DNA binding; dark grey, oligomerization. Equal volumes of cultures at the same optical density from each strain were spotted onto selective agar plates and grown for 2 days at the indicated temperatures. (B) Representative cells from wild-type (HSF) and HSF(1-583) cultures grown at 30°C or shifted to 37°C for 6 h were processed for Nomarski microscopy to determine cell morphology and DAPI staining to display DNA and photographed. Bar, 10 μm. (C) Distribution of cell morphologies at the 6-h point from the same population of cells depicted in panel B. The categories represent unbudded, small-budded (buds less than half the volume of the mother cell), and large-budded cells, respectively. (D) Flow cytometry of propidium iodide-stained wild-type and HSF(1-583) cells was carried out from culture aliquots removed at the indicated time points after a shift from 30 to 37°C. 1N and 2N refer to cells with haploid and diploid nuclei, respectively.

notype, early-log-phase cultures of isogenic wild-type (HSF) and HSF(1-583) cells were shifted from 30 to 37°C and cell morphology and nuclear status were monitored by Nomarski and DAPI fluorescence microscopy, respectively. Representative cells from culture aliquots taken immediately after the shift (0) and after 6 h at 37°C are shown in Fig. 1B. This time point is approximately when HSF(1-583) cultures exhibit a reduction in growth rate at 37°C, after undergoing two to three cell divisions at the restrictive temperature (data not shown). Wild-type cells remained unaffected by this treatment, continuing to bud, to segregate replicated nuclei, and to undergo cytokinesis without a measurable change in growth rate (Fig. 1B, top panels, and data not shown). Although HSF(1-583) cells displayed characteristics similar to those of the wild-type strain at 30°C, at 37°C a high percentage (80%) of the cells accumulated as oversized, large-budded cells (Fig. 1B and C). DAPI staining of these same cells revealed that each large-budded cell possessed only a single nucleus, consistent with arrest early in the G_2/M phase of the cell cycle. Interestingly, the remainder of the population (20%) consisted almost entirely of unbudded cells, suggesting that a small fraction of the cells were experiencing a G_1/S-phase arrest (Fig. 1C).

To further delineate the cell cycle arrest point, aliquots of HSF and HSF(1-583) cultures were removed at 0, 3, and 6 h after shift to 37°C and stained with propidium iodide for flow cytometric analysis of DNA content. As shown in Fig. 1D, HSF(1-583) cells displayed a normal distribution of cells with 1N and 2N DNA content, representing pre- and post-S-phase cells, respectively, both immediately and 3 h after the shift to 37°C. In contrast, after 6 h at 37°C the HSF(1-583) culture was predominantly composed of G_2-phase cells with replicated (2N) nuclear material. Microscopic examination of the same propidium iodide-stained cells analyzed by flow cytometry revealed that these were large-budded cells that contained only a single nucleus (data not shown). Wild-type control cells did not exhibit this phenotype, maintaining a similar distribution of G_1- and G_2-phase cells throughout the incubation (Fig. 1D). As further evidence that the temperature-sensitive growth defect is a bona fide cell cycle arrest rather than increased temperature lethality, HSF(1-583) cells could be maintained at 37°C for up to 24 h before exhibiting reduction in cell viability upon return to the permissive growth temperature of 30°C (data not shown).

Cell cycle arrest due to loss of the HSF CTA is suppressed by multiple copies of a yeast Hsp90 gene. Although the HSF CTA plays an important role in *CUP1* expression in response to heat stress, *cup1*Δ strains display no cell cycle or temperature-sensitive phenotypes (22). Because HSF coordinates the expression of a number of heat-inducible genes, we reasoned that loss of the CTA might block the production of one or more gene products crucial to cell cycle progression at 37°C. To test this hypothesis, a selection for genes which could suppress the temperature-sensitive growth defect of HSF(1-583) cells when expressed from a 2μm high-copy vector was carried out. A strain harboring HSF(1-583) as the only functional HSF was transformed with a genomic library and plated on selective medium. From approximately 60,000 independent transformants screened, six plasmids which conferred growth at 37°C were recovered. These six were placed into classes based on restriction digestion patterns, and representative clones were sequenced from both ends of the insert to identify the genomic fragments present. BLAST (2) searches of the *Saccharomyces* genome database (14) revealed that three of the plasmids carried an identical insert which contained the *HSF1* locus encoding full-length HSF and that three others contained nonidentical but overlapping DNA fragments as depicted in Fig. 2A. Analysis of the region common to both genomic inserts revealed two potential candidates for high-copy suppressors of HSF(1-583) temperature sensitivity: the *CIN2* gene, which encodes a protein involved in microtubule function (25, 60), and the *HSP82* gene, which encodes the heat-inducible isoform of the Hsp90 family of heat shock proteins (19). Because *cin2*

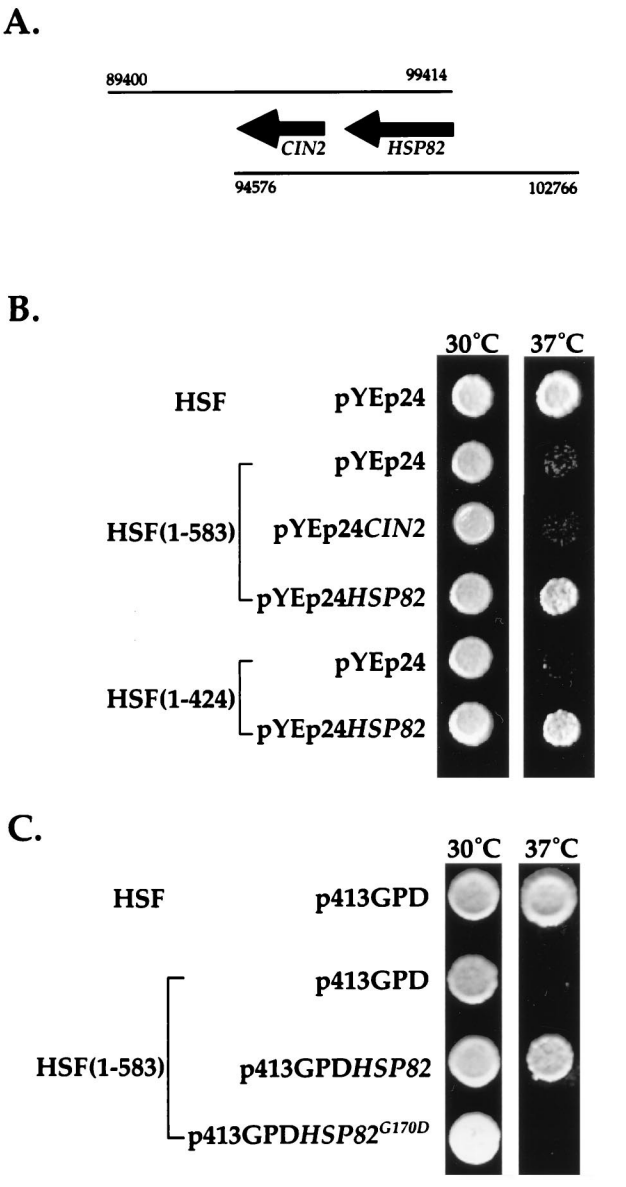

FIG. 2. Cell cycle arrest of HSF(1-583) cells can be suppressed by functional *HSP82*. (A) The two classes of genomic inserts within the suppressing plasmids are shown with endpoints corresponding to coordinates obtained from the *Saccharomyces* genome database (14). The two open reading frames located within the overlapping region are identified as *CIN2*, a gene involved in microtubule function, and *HSP82*, encoding a heat-inducible Hsp90 isoform. (B) Subcloning and suppression analysis of *CIN2* and *HSP82* identifies the Hsp90 gene *HSP82* as the suppressing locus for the temperature sensitivity of the HSF(1-583) mutation. Multicopy *HSP82* also effectively suppresses the temperature sensitivity of an isogenic strain bearing the HSF(1-424) allele. (C) Plasmids containing either wild type or the G170D mutant alleles of *HSP82* expressed from the constitutive GPD promoter were transformed into HSF(1-583) cells. The empty vector alone was transformed into both HSF and HSF(1-583) cells as controls. Aliquots of the indicated strains were serially diluted and spotted to selective agar medium for the HSF(1-583) suppression assay as described in the legend to Fig. 1.

mutants arrest just prior to mitosis (25), analogous to our findings with HSF(1-583) cells, both genes were potentially relevant for suppression of the G_2/M arrest. To identify which gene was responsible for suppression, DNA fragments encoding either gene were subcloned by restriction digestion to fresh YEp24 vectors and the plasmids were introduced into HSF(1-583) cells and independently assayed for their ability to confer growth at 37°C (Fig. 2B). YEp24*HSP82* clearly allowed growth of HSF(1-583) cells, while the YEp24*CIN2* plasmid did not, localizing multicopy suppression to the *HSP82* locus. Furthermore, YEp24*HSP82* also suppressed the temperature-sensitive growth phenotype of HSF(1-424) cells (Fig. 2B), suggesting that the phenotype of both HSF truncation alleles is due to a common defect. We noted that suppression of HSF(1-583) by high-copy *HSP82* was incomplete, as evidenced by smaller, less robust colonies at 37°C and by a growth rate in liquid cultures at 37°C intermediate between those of wild-type and HSF(1-583) strains (40). Although the *HSP82* gene was isolated as a high-copy suppressor of strain PS145*HSF(1-583), expressing the HSF(1-583) allele from a centromeric plasmid, suppression was also observed in strain DTY179, the HSF(1-583) strain characterized for Fig. 1 (40). Consistent with their ability to grow at 37°C, these cells did not undergo cell cycle arrest when shifted to the restricted temperature (40).

The Hsp90 class of heat shock proteins is known to possess protein chaperone activity, as demonstrated by the ability of Hsp90 to prevent the aggregation of purified model substrates such as unfolded citrate synthase (67) and casein kinase II (38). Furthermore, in concert with Hsp70 and ATP, Hsp90 participates in the refolding and activation of firefly luciferase in vitro (51) and is also required for full activity after de novo synthesis in vivo (44). A mutant allele of *HSP82*, encoding an Hsp90 protein with a glycine-to-aspartic acid substitution in residue 170 (G170D), has been independently isolated three times as a temperature-conditional mutation (6, 32, 43). This mutation maps within the highly conserved ATP-geldanamycin binding pocket located in the amino terminus of the protein (21, 48, 61). *S. cerevisiae* cells harboring this allele as their only source of Hsp90 are fully competent for growth at normal temperatures but are temperature sensitive due to the rapid inactivation of Hsp82p^{G170D} after a shift to temperatures at or greater than 34°C (43). In addition, this mutant was recently demonstrated to be deficient in folding of newly synthesized firefly luciferase expressed in yeast (44). To test whether the protein chaperone activity of Hsp90 was required for cell cycle progression during stress, the ability of HSF(1-583) cells carrying the *HSP82*^{G170D} allele to grow at the restrictive temperature of 37°C was assessed. When expressed from the strong GPD promoter, *HSP82*^{G170D} failed to suppress the temperature sensitivity of HSF(1-583) (Fig. 2C). Increasing the expression of this mutant allele by placing it on a high-copy-number 2μm-based vector was not sufficient to restore growth, indicating that the defect incurred by the G170D mutation cannot be overcome in a dosage-dependent manner (40). Therefore, restoration of cell cycle progression in HSF(1-583) cells at stress temperatures by Hsp82p required functional activity of the protein, which is abrogated in the *HSP82*^{G170D} mutant at 37°C.

Suppression of the HSF(1-583) cell cycle block is specific to HSP82 and reveals a functional distinction between the two yeast Hsp90 isoforms. *S. cerevisiae* harbors two genes which encode 97% identical forms of the heat shock protein Hsp90. The *HSC82* gene is constitutively expressed and displays approximately twofold induction upon heat shock (8). In contrast, the *HSP82* gene product is not detected during growth under nonstress conditions and is highly expressed during heat shock (8). The basal and heat shock-induced expression of both genes is controlled by HSF and *cis*-acting heat shock elements (18, 37). Previous studies have shown that deletion of both genes is lethal, while deletion of either one independently results only in a moderate growth defect at elevated temperatures (8). We isolated genomic DNA fragments encompassing *HSP82* three times in our suppressor screen but failed to isolate *HSC82*, suggesting that either *HSC82* is underrepresented in the genomic library we employed or multicopy *HSC82* is incapable of complementing the HSF(1-583) defect. To distinguish between these possibilities, a plasmid which carries *HSC82* in the same vector utilized in the genomic library was constructed to ensure equivalent levels of overexpression. Surprisingly, while HSF(1-583) cells harboring plasmid pYEp24*HSP82* grew at 37°C, those transformed with pYEp24*HSC82* failed to suppress the temperature sensitivity of the HSF(1-583) strain (Fig. 3A).

To verify that cells harboring these plasmids expressed the respective Hsp90 protein, whole-cell protein extracts were prepared from cultures grown at 30°C or heat shocked for 1 h at 39°C, and levels of Hsp90 were determined by immunoblotting. By using polyclonal antiserum which recognizes both Hsc82p and Hsp82p, a polypeptide which was present at moderate levels at control temperatures but highly induced as a result of heat shock in wild-type cells was detected (Fig. 3B, lanes 1 and 2). Based on previous reports, the polypeptide present at control temperatures corresponds to Hsc82p, and the protein detected from heat-shocked cells is primarily Hsp82p (8). Expression of both Hsp90 isoforms was severely diminished in HSF(1-583) cells, demonstrating, as for *CUP1*, that the HSF CTA is required for both basal and heat-induced expression of these two genes (Fig. 3B, compare lanes 3 and 4 to lanes 1 and 2, respectively). Expression of both Hsc82p and Hsp82p was greatly increased in strain HSF(1-583) transformed with YEp24*HSC82* or YEp24*HSP82* (Fig. 3B, lanes 5 to 8). Interestingly, proper gene regulation was maintained in cells containing the YEp24*HSP82* plasmid, as expression of Hsp82p remained heat inducible. Moreover, the levels of both Hsc82p and Hsp82p in these strains were nearly identical, as judged by densitometric quantitation, at approximately 25-fold over their respective levels in HSF(1-583). Suppression of the G_2/M arrest at 37°C in HSF(1-583) by *HSC82* was observed, however, when the gene was placed under the constitutive GPD promoter (42), which produced Hsc82p at approximately fourfold-higher levels than that expressed from YEp24*HSC82* (Fig. 3C and data not shown). Taken together, these data demonstrate that the heat-inducible Hsp82p aids progression through the cell cycle during thermal stress in the HSF(1-583) background more efficiently than the closely related Hsc82p.

HSF(1-583) cells are hypersensitive to the antitumor drug geldanamycin. The benzoquinoid ansamycins are a class of naturally occurring antibiotics which have been shown to reverse cellular transformation by oncogenic tyrosine kinases such as pp60$^{v\text{-}src}$ (15, 65). Initially thought to act as protein tyrosine kinase inhibitors, these compounds are now known to pharmacologically block the interaction of substrates with Hsp90, an interaction required for their stability and function (66). The most potent of these agents is geldanamycin, currently in preclinical development as an antitumor drug by the National Cancer Institute (63). Geldanamycin has been demonstrated to bind to both mammalian and chicken Hsp90 (21, 66), and the cocrystal structure of a bovine Hsp90-geldanamycin complex has recently demonstrated that the drug binds within a conserved pocket which also constitutes the nucleotide binding site of the molecule, as determined for yeast Hsp82p (48, 61). Consistent with the in vitro functional data and structural implications, refolding of denatured firefly luciferase by

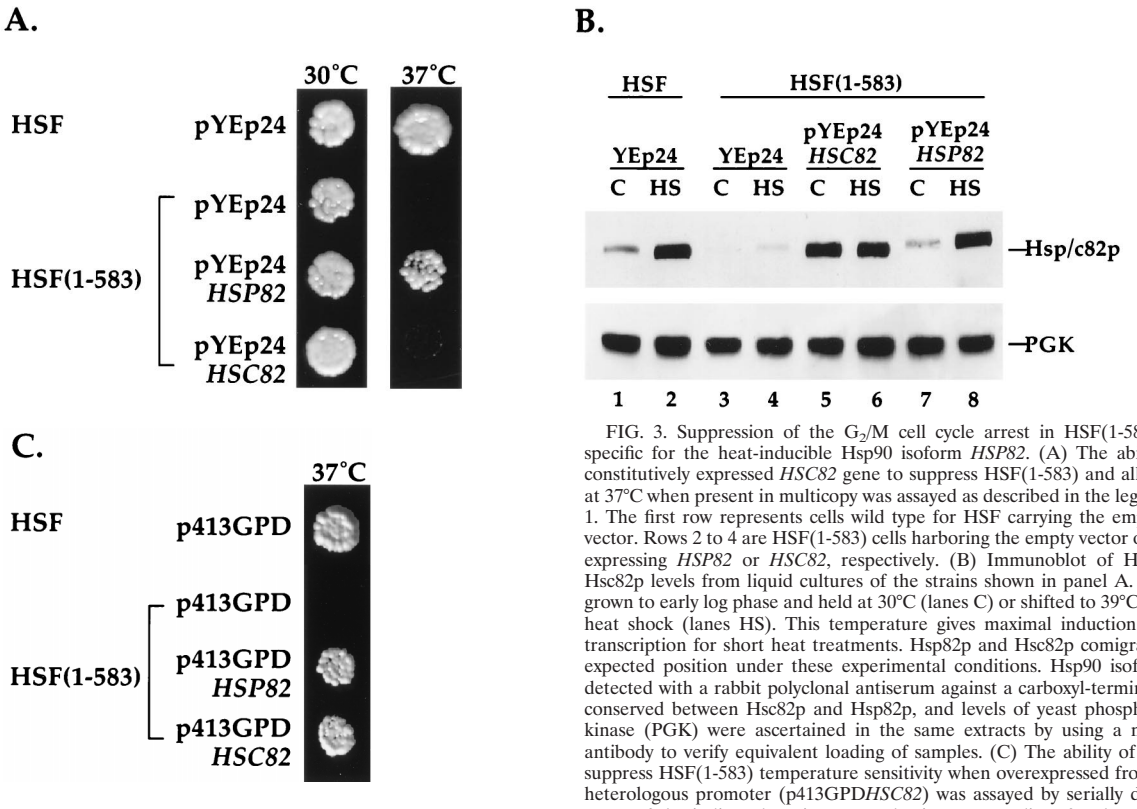

FIG. 3. Suppression of the G_2/M cell cycle arrest in HSF(1-583) cells is specific for the heat-inducible Hsp90 isoform *HSP82*. (A) The ability of the constitutively expressed *HSC82* gene to suppress HSF(1-583) and allow growth at 37°C when present in multicopy was assayed as described in the legend to Fig. 1. The first row represents cells wild type for HSF carrying the empty YEp24 vector. Rows 2 to 4 are HSF(1-583) cells harboring the empty vector or plasmids expressing *HSP82* or *HSC82*, respectively. (B) Immunoblot of Hsp82p and Hsc82p levels from liquid cultures of the strains shown in panel A. Cells were grown to early log phase and held at 30°C (lanes C) or shifted to 39°C for 1 h for heat shock (lanes HS). This temperature gives maximal induction of *HSP82* transcription for short heat treatments. Hsp82p and Hsc82p comigrated at the expected position under these experimental conditions. Hsp90 isoforms were detected with a rabbit polyclonal antiserum against a carboxyl-terminal epitope conserved between Hsc82p and Hsp82p, and levels of yeast phosphoglycerate kinase (PGK) were ascertained in the same extracts by using a monoclonal antibody to verify equivalent loading of samples. (C) The ability of Hsc82p to suppress HSF(1-583) temperature sensitivity when overexpressed from a strong heterologous promoter (p413GPD*HSC82*) was assayed by serially diluting aliquots of the indicated strains onto selective agar medium for the HSF(1-583) suppression assay as described in the legend to Fig. 1.

the Hsp90 chaperone complex is also inhibited by treatment with ansamycins, which prevent release of the substrate, confirming that the drug plays a role in Hsp90 chaperone function (51). Additionally, geldanamycin has been shown to inhibit ligand-dependent transcriptional activation by the mineralocorticoid receptor and GR in a dose-dependent manner, further demonstrating the necessity of Hsp90 function for activation of this class of transcription factors (3, 47, 54).

To determine if the reduction of Hsp90 levels in an HSF(1-583) background might predispose cells to geldanamycin sensitivity, we developed a growth assay for the effects of geldanamycin in yeast (Fig. 4A). Though geldanamycin is normally effective in the low-nanomolar range against human cancer cell lines (63), we found that growth was unimpaired in cells expressing wild-type HSF at concentrations of 2 mM geldanamycin. In contrast, HSF(1-583) cells displayed a large zone of growth inhibition in the presence of geldanamycin, which was absent when cells were treated with the solvent, DMSO, alone. This sensitivity of HSF(1-583) cells, but not wild-type cells, was exacerbated at slightly elevated temperatures, which do not normally affect HSF(1-583) growth (data not shown). Microscopic examination of the zone of inhibition revealed that the majority of cells underwent one to two divisions before ceasing growth, consistent with the phenotype reported for strains with deletions of both *HSC82* and *HSP82* (Fig. 4A, 25×) (8). Moreover, accumulation of large-budded cells was not observed, in accordance with the G_2/M cell cycle arrest of HSF(1-583) cells being a heat shock-dependent phenotype. Importantly, restoration of Hsp90 levels in HSF(1-583) cells through ectopic expression of either *HSC82* or *HSP82* conferred resistance to geldanamycin and allowed colony formation to a level indistinguishable from that of wild type, consistent with the high degree of conservation of the predicted geldanamycin binding pocket in both Hsc82p and Hsp82p (48). These data demonstrate that yeast cells compromised for Hsp90 expression, via a defect in the HSF carboxyl-terminal transactivation domain, are hypersensitive to geldanamycin. Furthermore, amelioration of geldanamycin sensitivity is not selective for either Hsp90 isoform, providing further evidence that the requirements for Hsp90 function for growth during normal or heat stress conditions are not identically provided by Hsc82p and Hsp82p, respectively.

Because low levels of Hsp90 also compromise the activation potential of hormone receptors in yeast (46), we tested whether hormone receptor function in HSF(1-583) cells would be hypersensitive to geldanamycin inhibition relative to HSF wild-type cells. Isogenic HSF and HSF(1-583) strains were transformed with plasmids constitutively expressing the rat GR and a glucocorticoid response element-*lacZ* (GRE-*lacZ*) reporter gene. Wild-type and HSF(1-583) cells harboring the GR reporter system were divided into equal aliquots and pretreated with the indicated concentrations of geldanamycin for 30 min. Each geldanamycin-treated sample culture was then further divided in two and treated with either ethanol alone or the synthetic hormone analog DOC (10 μM) for an additional 60 min. To precisely determine the effects of geldanamycin treatment, the levels of *lacZ* mRNA produced during hormone induction were measured by RNase protection assays and normalized to *ACT1* mRNA levels, as shown in Fig. 4B and quantitated in Fig. 4C. In the absence of geldanamycin, DOC-dependent transcription of GRE-*lacZ* was approximately equivalent, at 30-fold above noninduced levels in both wild-

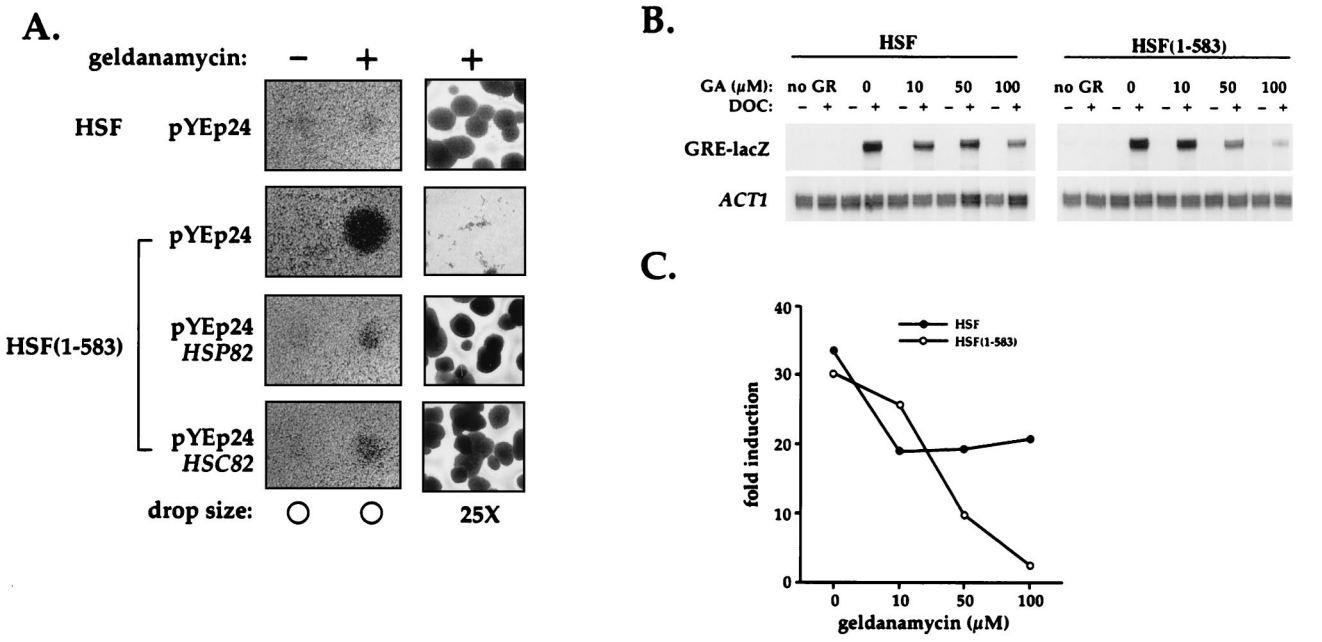

FIG. 4. HSF(1-583) cells are hypersensitive to geldanamycin. (A) Low-density lawns of the indicated isogenic strains were treated with geldanamycin (+) or DMSO alone (−) as described in detail in Materials and Methods. The circles below the panels show the location and diameter of the 10-μl drug aliquot applied to the top agar. Plates were incubated for 3 days at 30°C and photographed directly or under low-power magnification (25×) to more accurately discern microcolony formation. Inhibition of growth by geldanamycin in the left panels results in a dark zone within the white background of confluent cells, while growth seen under magnification (25×) is manifest as dark colonies against a light grey background. (B) Wild-type and HSF(1-583) cells carrying pYRP-GRElacZ and either pRS413GPD (no GR) or pRS413GPDGR were grown at 30°C and treated for 30 min with the indicated concentrations of geldanamycin (GA), followed by an additional 60 min of incubation in the presence (+) or absence (−) of the steroid hormone analog DOC. RNase protection was carried out on total RNA with radiolabeled probes hybridizing with *lacZ* (GRE-lacZ) and *ACT1* mRNA. (C) Phosphorimage quantitation of the data presented in panel A. Fold induction is calculated as the ratio of the intensity of the *lacZ* signal normalized to *ACT1* from DOC-treated and untreated samples.

type and HSF(1-583) cells. GR function in wild-type cells was slightly reduced in the presence of 10 μM geldanamycin, giving an approximately 20-fold level of induction, but was not further affected by increasing concentrations of the drug. In contrast, HSF(1-583) cells displayed a dose-dependent decrease in steroid-induced reporter gene transcription, exhibiting less than threefold activation in the presence of 100 μM geldanamycin. In both strain backgrounds, the levels of *ACT1* mRNA used as a control were unaffected by geldanamycin administration. With the GR, a well-characterized Hsp90 substrate protein, these data confirm that HSF(1-583) cells are deficient in Hsp90 function due to decreased Hsp90 levels, as reflected in increased sensitivity to the Hsp90 inhibitor geldanamycin.

Specific depletion of Hsp90 during heat stress results in cell cycle arrest. The identification of *HSP82* as a multicopy suppressor of HSF(1-583) temperature sensitivity and the dramatically low levels of both yeast Hsp90 isoforms in this strain together argue for an important role for Hsp90 in cell cycle progression at 37°C. Strains which lack functional Hsp90 due to chromosomal gene disruptions in both *HSC82* and *HSP82* are inviable, and experiments involving regulated depletion of Hsp90 at normal growth temperatures have not revealed a cell cycle arrest phenotype similar to that we have described at 37°C (30). To further test whether loss of Hsp90 represents a critical determinant of the cell cycle arrest in HSF(1-583) cells during thermal stress, we depleted Hsp82p in an HSF wild-type strain under conditions which closely paralleled the arrest of HSF(1-583) cells. Strain 5CG2 contains a chromosomal disruption in the *HSC82* gene, and the endogenous *HSP82* gene is replaced by a modified allele in which expression is under the regulation of the *GAL1* promoter (30, 46). This promoter allows regulation of Hsp82p production by carbon source: growth on galactose induces expression while glucose represses transcription, as evidenced by the inviability of strain 5CG2 in standard glucose medium (30, 46). 5CG2 cells were grown to log phase at 30°C in galactose (t_0) and then shifted to glucose for 5 h to initiate depletion of Hsp82p (t_5; Fig. 5A). After 5 h of continued growth (presumably due to preexisting levels of Hsp82p), the culture was transferred to 37°C for 10 additional h (t_{15}). An immunoblot of whole-cell protein extracts from culture aliquots taken at these time points revealed that levels of Hsp82p dropped precipitously from initiation of the experiment to the 5-h point (Fig. 5B). Longer exposures of the blot established that low levels of Hsp82p were still present at this time, consistent with previous reports that very little functional Hsp90 is required for yeast growth under normal conditions (reference 46 and data not shown). No Hsp82p was detected at the 15-h point at any exposure by immunoblotting. Fluorescence microscopy of DAPI-stained cells from these same time points revealed that while nuclear positioning and cell morphology were normal at 30°C, cells taken at the 15-h point showed a mix of enlarged unbudded and large-budded cells (Fig. 5C, t_{15}). A budding index calculated from this sample revealed an approximately equal distribution of unbudded and large-budded cells, with essentially no cells displaying the small-budded morphology characteristic of actively growing cultures (Fig. 5C, compare panel t_{15} to panels t_0 and t_5). Control cultures maintained at 30°C throughout the same time course did not exhibit this same phenotype but instead ceased growth and produced cells with elongated and multiple-budded morphologies, consistent with previous reports (reference 30 and data not shown). Therefore, depletion of Hsp90 in HSF

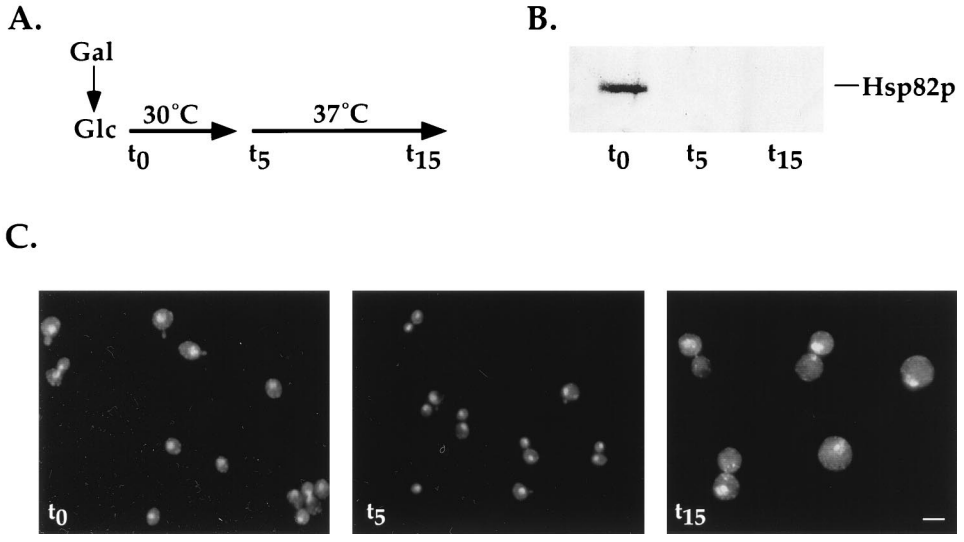

FIG. 5. Specific depletion of Hsp90 during heat shock results in cell cycle arrest. (A) Schematic depiction of the depletion experiment. 5CG2 cells (relevant genotype *hsc82 hsp82::GAL1HSP82*) were grown overnight in galactose medium, then shifted to glucose medium (t_0) to repress *HSP82* transcription and allow depletion of Hsp82p for 5 h (t_5), and then shifted to 37°C for an additional 10 h (t_{15}). Aliquots of the culture were drawn at the indicated time points, and whole-cell protein extracts were made for immunoblot analysis with anti-Hsp90 antiserum (B) or microscopic examination of DNA by DAPI staining (C). Bar, 10 μm.

wild-type cells also resulted in cell cycle progression defects during thermal stress, in this case giving rise to a larger fraction of the cell population arrested in G_1 (50%) compared to the arrest observed in the HSF(1-583) mutant (20%). While these data do not address the relative importance of *HSC82* versus *HSP82* in cell cycle progression, they strengthen the hypothesis that loss of Hsp90 expression is a key determinant in cell cycle arrest of HSF(1-583) cells, by demonstrating that loss of Hsp90 alone is sufficient to cause arrest. Furthermore, we propose that the different ratios of G_1/S- and G_2/M-phase cells observed after heat shock of HSF(1-583) cells or *GAL1-HSP82* depletion may be due to other gene targets of HSF whose expression is affected in the HSF(1-583) mutant.

DISCUSSION

A great deal of work has demonstrated that Hsp90 functions as a protein chaperone in vitro and is required for the stability, function, and localization of protein kinases and steroid hormone receptors and other proteins involved in cell regulation. However, no substrates have been directly demonstrated to require Hsp90 for function in *S. cerevisiae* (27). This observation is inconsistent with the explicit necessity for Hsp90 for survival and the presence of two separate genes, one constitutively expressed (*HSC82*) and one strongly heat inducible (*HSP82*), encoding Hsp90 isoforms in yeast. Here we demonstrate that removal of the HSF carboxyl-terminal transactivation domain renders cells temperature sensitive for growth during thermal stress via a reversible cell cycle arrest in the G_2/M phase of the cell cycle. Restoration of *HSP82* expression to wild-type levels permits cell cycle progression at 37°C, while *HSC82* must be expressed at higher levels to achieve the same effect. In this context, the isolation of *HSP82* as a multicopy suppressor can be accounted for by two distinct explanations: (i) HSF(1-583) is deficient in expression of *HSP82*, which may itself be crucial for cell cycle progression during stress, or (ii) overexpression of a ubiquitous protein chaperone may be bypassing loss of another gene product required for cell cycle progression whose expression is directly or indirectly controlled by HSF. While the data are consistent with both scenarios, it is unlikely that multicopy suppression of HSF(1-583) temperature sensitivity by *HSP82* is an artifact of the genetic selection for the following reasons. First, immunoblot analysis of extracts from control and heat-shocked HSF(1-583) cells showed that protein levels of Hsp90 were severely reduced in comparison to those of wild-type cells. Consistent with this observation, we have determined that transcription of both the *HSC82* and *HSP82* genes is dramatically reduced in HSF(1-583) cells (50). Second, HSF(1-583) cells bearing the YEp24*HSP82* plasmid expressed Hsp82p levels which were nearly identical to that seen in wild-type cells, suggesting that expression of *HSP82* by an increase in gene copy number compensated for lowered transcription efficiency but did not result in gross overproduction of Hsp90. Third, overexpression of the Hsp70 chaperone gene *SSA1* or *SSA3* from high-copy vectors failed to suppress the temperature sensitivity of HSF(1-583) cells (40). Finally, we demonstrated that depletion of Hsp90 alone from otherwise wild-type cells largely recapitulated the cell cycle arrest phenotype of HSF(1-583). Together, these results argue for a direct role for the Hsp90 protein chaperone in cell cycle progression during thermal stress.

Since the identification of two closely related yeast genes encoding isoforms of Hsp90, no functional differences have been observed between them as judged by a variety of experimental criteria. For example, either gene alone can support growth at wild-type rates during normal conditions with only moderate growth defects at higher temperatures (8). In addition, both Hsc82p and Hsp82p are capable of forming complexes with mammalian substrates such as $pp60^{v\text{-}src}$ or the glucocorticoid steroid hormone receptor expressed in yeast (11, 43, 46). However, we find that at comparable levels of expression, only the *HSP82* gene product is capable of restoring cell cycle progression to HSF(1-583) cells at 37°C. This result is consistent with previous characterization of Hsp82p as a strongly heat-inducible yeast Hsp90 isoform and may reflect either a greater need for the Hsp90 chaperone at stress temperatures or specific attributes of Hsp82p. Both Hsp82p and Hsc82p are highly conserved (97% identity) at the primary sequence level, with only 12 amino acid substitutions located throughout the length of the protein (8). In addition, Hsp82p

contains five repeats of a highly conserved region of repeated acidic and basic residues, whereas only four repeats are found within Hsc82p. The functional significance of this unusual sequence is not clear, however, as it is not found in the *Escherichia coli* Hsp90 homolog *htpG* (4) and is dispensable for yeast viability (36). Since increasing the level of Hsc82p severalfold above that of Hsp82p by artificially overexpressing the *HSC82* gene from a strong constitutive promoter also allowed suppression of HSF(1-583), we favor a model which incorporates both possibilities: the requirement for Hsp90 function at 37°C can be potentially fulfilled by either yeast isoform but is preferentially selective for Hsp82p, which may possess subtle substrate specificity relative to Hsc82p and is genetically programmed for rapid production during heat shock conditions. This hypothesis is compatible with the apparent prodigality of having a second, heat-inducible isoform of Hsp90 when the *HSC82* gene is already highly expressed (up to 1% of total cell protein by some estimates in yeast [8]).

An intriguing component of this work is the identification of a cell cycle arrest phenotype at stress temperatures for cells harboring a partially functional HSF molecule. A temperature-sensitive allele of *HSF1*, *mas3*, was previously reported to undergo a similar cell cycle arrest, but the precise nature of HSF produced from this mutant and its effects on heat shock protein synthesis are not clear (55). Recently, an additional uncharacterized mutant *HSF1* allele was recently found to be synthetically lethal with a novel G_2/M-arresting allele of *CDC28*, the yeast cyclin-dependent kinase required for cell cycle progression (72). This mutant, *hsf1-82*, exhibits reduced expression of Hsp90 and also shows defects in progression through both the G_1/S and G_2/M phases of the cell cycle. Characterization of spindle pole body (SPB) formation by electron microscopy revealed that SPB duplication, as well as spindle elongation, was perturbed in this mutant (72). Moreover, we determined that the G_2/M-phase arrest of HSF(1-583) was characterized by mixed populations of cells containing either a monopolar SPB or duplicated SPBs connected by a short premitotic spindle by antitubulin indirect immunofluorescence microscopy (40). At this point, it is not clear what the primary targets of Hsp90 are in SPB morphogenesis, as both studies have highlighted multiple stages of arrest which may not be attributable to malfunction of a single known protein. A number of gene products whose function is required for SPB assembly and duplication have been identified, and defects in spindle morphogenesis lead to transient G_2/M-phase arrest by activation of a mitotic checkpoint. If Hsp90 or other gene products controlled by HSF are required for stability or function of one or more of these proteins under conditions of thermal stress, then defects in SPB assembly or morphology which activate this checkpoint may ensue.

A link between chaperone function and cell cycle progression during stress is consistent with recent findings which implicate Hsp90 and subunits of the Hsp90 chaperone complex with proteins involved in cell cycle control. In the yeast *Schizosaccharomyces pombe*, Hsp90 is required for the stability and activity of the cell cycle regulator Wee1 (1). The Cdc37 protein has recently been shown to function as a protein chaperone in vitro (31, 62), and yeast *CDC37* genetically interacts with both the *MPS1* (52) and *CDC28* (20) kinases involved in the spindle assembly checkpoint and control of the cell cycle, respectively, as well as mammalian $pp60^{v\text{-}src}$ expressed in yeast (16). Indeed, Cdc37p has been characterized as a "protein kinase chaperone" which may target kinases for recognition and regulation by the Hsp90 chaperone complex (26). Similarly, *YDJ1* displays synthetic lethality with mutant alleles of *CDC28* (72) and *MPS1* (53). Moreover, specific alleles of *CDC28* and *CDC37*, both initially identified as mutants which arrested early in the cell cycle at START (24), also arrest at the G_2/M transition phase (16, 72), providing a potential rationale for the mixed population of Hsp90-depleted cells arresting at G_1/S and G_2/M depicted in Fig. 5. Further genetic and biochemical work will be required to dissect the phase-specific requirements for the Hsp90 chaperone complex in cell cycle progression during heat stress. The identification of a physiological requirement for HSF, and specifically the carboxyl-terminal transcriptional activation domain, function in yeast cell cycle progression, however, represents an important advance in the search for roles for the heat shock response in cellular processes during stress.

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