Requirement for Hsp90 and a CyP-40-type Cyclophilin in Negative Regulation of the Heat Shock Response

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The heat shock response is a highly conserved mechanism that allows cells to withstand a variety of stress conditions. Activation of this response is characterized by increased synthesis of heat shock proteins (HSPs), which protect cellular proteins from stress-induced denaturation. Heat shock transcription factors (HSFs) are required for increased expression of HSPs during stress conditions and can be found in complexes containing components of the Hsp90 molecular chaperone machinery, raising the possibility that Hsp90 is involved in regulation of the heat shock response. To test this, we have assessed the effects of mutations that impair activity of the Hsp90 machinery on heat shock related events in Saccharomyces cerevisiae. Mutations that either reduce the level of Hsp90 protein or eliminate Cpr7, a CyP-40-type cyclophilin required for full Hsp90 function, resulted in increased HSF-dependent activities. Genetic tests also revealed that Hsp90 and Cpr7 function synergistically to repress gene expression from HSF-dependent promoters. Conditional loss of Hsp90 activity resulted in both increased HSF-dependent gene expression and acquisition of a thermotolerant phenotype. Our results reveal that Hsp90 and Cpr7 are required for negative regulation of the heat shock response under both stress and nonstress conditions and establish a specific endogenous role for the Hsp90 machinery in S. cerevisiae.

All cells possess a defense mechanism known as the heat shock response, which allows them to survive exposure to otherwise lethal doses of certain stresses (1–3). These stresses include environmental challenges, such as elevated temperatures, and pathophysiological states, such as viral infections (4). The heat shock response is characterized by increased synthesis of a set of proteins collectively referred to as heat shock proteins (HSPs) whose principal role is to assist target substrates in their synthesis, transport, and proper folding (5–7). The requirement for chaperoning activities increases as cells are exposed to elevated temperatures or to other conditions that promote protein denaturation and aggregation. Because chaperoning activity is also crucial for the function of proteins not exposed to stress, HSPs play important roles for life under normal conditions as well.

Expression of HSPs is under the control of heat shock transcription factors (HSFs) (4, 8, 9). In most eukaryotic systems, HSF is maintained as a monomer unable to bind DNA until activated by stress (4, 8). Activated HSF forms homotrimeric capable of binding to heat shock elements (HSEs) present at promoters of genes encoding HSPs, ultimately leading to transcriptional activation (4, 8). The monomer to trimer transition is believed to be negatively regulated, at least in part, by Hsp70 (4, 8). The acquisition of transcriptional activity by HSF is correlated with increased phosphorylation (10); however, the functional relationship between phosphorylation and regulation of the heat shock response is still not fully understood.

In contrast to most eukaryotic cells, in Saccharomyces cerevisiae HSF is bound to HSEs even in the absence of stress (11). This observation led to the proposal that yeast HSF bypasses the monomer to trimer and DNA binding regulatory steps. However, subsequent work has shown that heat shock treatment leads to increased HSE occupancy by HSF at the yeast HSP82 promoter (12), suggesting that regulation of HSF activity in yeast is mediated in part by conversion of HSF from a non-DNA binding form to one competent to bind HSEs.

Perhaps the best characterized aspect of HSF regulation in S. cerevisiae comes from investigation of the functional relationship between Hsp70 and the heat shock response. Mutations that decrease Hsp70 levels confer increased expression of several HSPs and constitutive thermotolerance (13, 14). Furthermore, these mutants exhibit a slow growth phenotype that can be suppressed by a mutation in HSF that decreases function of the transcription factor (15). These and other results from both mammalian and yeast systems, including the observation that the heat shock response is transient in nature, have led to the proposal for an autoregulatory loop in which Hsp70 normally represses HSF activity. According to this model, during heat shock Hsp70 dissociates from HSF, resulting in a net increase in synthesis of HSPs, including Hsp70 itself. Elevated levels of Hsp70 in turn lead to increased binding of the chaperone to HSF resulting in repression of HSF and subsequent down-regulation of the response (4, 8, 16).

In addition to Hsp70, other molecular chaperones have been proposed to be involved in regulation of the heat shock response (9). A recent study has shown that the mammalian cochaperone Hdj1 is involved in negative regulation of HSF1 activity (17). Because yeast and mammalian HSF can physically associate with Hsp90 (18, 19), it is possible that the Hsp90 machinery also participates in regulation of the heat shock response. Furthermore, because Hsp70 and Hsp90 can be found together in the same protein complexes (20), some of the functions ascribed to Hsp70 in regulation of HSF activity may actually reflect a joint effect with Hsp90.

Hsp90 associates with several proteins, including the cyclophilin CyP-40, to form heterocomplexes that regulate the ac-

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1 The abbreviations used are: HSP, heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element.

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tivity of a number of cellular factors, such as steroid receptors and oncogenic tyrosine kinases (20, 21). More recently, mammalian Hsp90 has been shown to associate with endothelial nitric oxide synthase and to facilitate activation of the synthase in response to different signals (22). Although S. cerevisiae has been widely used to study the requirements for Hsp90 and Hsp90-associated proteins on activities of heterologous substrates (23–26), to date no endogenous role for Hsp90 has been identified in yeast. To test the possibility that the Hsp90 machinery is involved in regulation of the heat shock response, we have taken advantage of recently discovered mutations that significantly decrease the effectiveness of the Hsp90 machinery in S. cerevisiae and assessed their effects on HSF-dependent events. Our results show that Hsp90 and the yeast CyP-40 homolog Cpr7 are required for negative regulation of the heat shock response in yeast.

**EXPERIMENTAL PROCEDURES**

*Genetic Manipulation and Growth Media—* Standard genetic techniques and growth media used were those described in Kaiser et al. (27).

*Yeast Strains—* All S. cerevisiae strains used in this study derive from the W303 background. The hsc82Δ hsp82Δ hsp90Δ mutator strain, and cells deleted for HSC82 and/or CPR7 have been described in previous studies (24, 28–31).

**Assay for β-Galactosidase Activity—** The construction of the HSF-dependent pHSE2-lacZ and pZHJHSE2-26 reporter genes has been described previously (32, 33). Logarithmically growing wild-type and mutant cells (2.0 × 10^8 cells) harboring either reporter gene were harvested and resuspended in 350 μl of assay buffer (10 mM Tris, pH 7.3, 50 mM NaCl, 50 mM KCl, 10 mM MgCl_2, 20% w/v glycerol, 1 mM dithiothreitol, 0.4 μg/ml aprotinin (Sigma), 0.4 μg/ml leupeptin (Sigma), and 2 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma)). Cells were then lysed using acid-washed glass beads (B. Braun Biotech International) by vortexing at full speed for 30 s followed by incubation on ice for ~1 min (repeated for 4 cycles). Lysates were recovered, and β-galactosidase activity was measured by incubating the lysates in the presence of o-nitrophenyl-β-D-galactoside. Specific activity was determined using the following formula: A_420 = A_420_0 - A_420_t, where A_420 represents the extent of color development during the incubation with o-nitrophenyl-β-D-galactoside, A_420_0 represents the protein concentration of the lysates (determined using the Bio-Rad protein assay reagents), and v is the volume of lysate used in each reaction and t is the time elapsed during the reaction.

**Western Blot Analysis of Proteins—** Total cellular protein from logarithmically growing cells, obtained as described above, was resolved by SDS-polyacrylamide gel electrophoresis, transferred to solid support, and probed using antibodies specific to either Hsp104 or ribosomal protein L3. Immunostaining was performed using the ECL reagents (Amersham Pharmacia Biotech) following the manufacturer’s protocol.

**Assay for Acquired Thermotolerance—** Logarithmically growing cells cultured at 23 °C were either maintained at 23 °C or shifted to 33.5 or 37 °C for 1.5 or 3 h. Cells were then plated on solid medium prior to or following a 4-min 52 °C heat shock. Survival rates were calculated by extracting DNA from 1 ml of cells, resuspended in 350 μl of DNA extraction buffer (0.1 M Tris, pH 8.0, 1 M NaCl, 1% NP-40, 1 mM EDTA, 0.4 mg/ml aprotinin (Sigma), 0.4 μg/ml leupeptin (Sigma), and 2 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma)), and 2 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma). Cells were then lysed using acid-washed glass beads (B. Braun Biotech International) by vortexing at full speed for 30 s followed by incubation on ice for ~1 min (repeated for 4 cycles). Lysates were recovered, and β-galactosidase activity was measured by incubating the lysates in the presence of o-nitrophenyl-β-D-galactoside. Specific activity was determined using the following formula: A_420 = A_420_0 - A_420_t, where A_420 represents the extent of color development during the incubation with o-nitrophenyl-β-D-galactoside, A_420_0 represents the protein concentration of the lysates (determined using the Bio-Rad protein assay reagents), and v is the volume of lysate used in each reaction and t is the time elapsed during the reaction.

**RESULTS**

*Mutations That Decrease Hsp90 Function Result in Increased HSF-dependent Gene Expression—* Mammalian HSF1 can be found in complexes with the molecular chaperone Hsp90 and several Hsp90-associated proteins, including the cyclophilin CyP-40 (18). In addition, in S. cerevisiae HSF can be retained by affinity columns of purified yeast Hsp90 (19). To investigate the possibility that these interactions reflect a functional relationship between HSF and the Hsp90 chaperone machinery in vivo, we tested the effects of mutations that alter the Hsp90 machinery on the expression of an HSF-dependent reporter gene in S. cerevisiae. In these experiments, Hsp90 was impaired either by reducing the abundance of Hsp90 through deletion of HSC82 or by eliminating the cyclophilin Cpr7, a yeast CyP-40 homolog shown to interact directly with Hsp90 and to facilitate activation of the synthase (24). A plasmid harboring a synthetic promoter sequence containing two overlapping HSF binding sites (HSE2) placed upstream of a CYC-lacZ fusion (pHSE2-lacZ (32)) was introduced into wild-type, hsc82Δ, cpr7Δ, and hsc82Δ cpr7Δ cells, and the steady-state levels of β-galactosidase activity were measured. Compared with the activity from isogenic wild-type cells, β-galactosidase activity was 2.5-fold higher in cells deleted for HSC82 and ~11-fold higher in cells devoid of Cpr7 (Fig. 1A). Deletion of both HSC82 and CPR7 conferred a synergistic effect resulting in ~34-fold increase in β-galactosidase activity compared with wild-type cells (Fig. 1A), an increase comparable with that seen in wild-type cells subjected to conditions that fully activate the heat shock response (see Fig. 2). In contrast, none of the mutations affected the levels of β-galactosidase when CYC-lacZ was placed under the control of HSE12 (Fig. 1B), a mutant version of HSE2 that does not bind HSF (32). Thus, Hsp90 and Cpr7 specifically regulate HSF-dependent transcriptional activity. Although cpr7Δ and hsc82Δ cpr7Δ cells display a slow growth phenotype, the increase in reporter gene activity observed in these mutants is not because of a general

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3. HSC82, one of two genes that encode Hsp90 in S. cerevisiae, is responsible for 90% of Hsp90 protein level in cells grown under non-stress conditions (28).

4. Other investigators have reported a similar modest increase in HSF-dependent reporter gene expression in cells that harbor the hsc82Δ mutation (39).

5. A. A. Duina, H. M. Kalton, and R. F. Gaber, unpublished observation.
The negative regulation of the heat shock response by Hsp90 complex is involved in negative regulation of HSF activity even under conditions that elicit the full heat shock response. All cells were transformed with the pHSE2-lacZ reporter gene and assayed for β-galactosidase activity. Activity from wild-type cells cultured and maintained at 23 °C was compared with activities of wild-type and Hsp90 mutant cells (as indicated) subjected to conditions that fully elicit the heat shock response. Heat shock conditions (hs) were achieved by shifting cells cultured at 23 °C to a 39 °C water bath for 20 min and allowed to recover for 1 h at 23 °C. Indicated activities are relative to those obtained from wild-type cells not subjected to heat shock conditions. All data are means ± S.D. from two separate experiments in which at least two independent isolates of each genotype were tested.

A crucial aspect of activation of the heat shock response is the acquisition of a thermotolerant phenotype. We tested whether mutations in the Hsp90 machinery confer resistance to a sudden exposure to 52 °C for 4 min, a treatment that is normally lethal to cells that have not been allowed to mount a heat shock response.

Although β-galactosidase activity induced by pHSE2-lacZ is believed to reflect authentic HSF-mediated activity and has been widely used to study HSF regulation (32, 34, 35), we wished to authenticate the repressive effects of the Hsp90 machinery by monitoring the activity or abundance of proteins that are expressed from promoters harboring endogenous HSF-binding sites. In one set of experiments, a plasmid containing a fusion of the HSE2 element of the Hsp70-encoding gene (pZJHSE2–26 (33)) was introduced into wild-type and mutant cells and assayed for steady-state lacZ activity. The Hsp90 complex is involved in negative regulation of the heat shock response by Hsp90.

Effect conferred by slow growth because cells deleted for END4, a gene encoding a factor involved in endocytosis, grow slower than hsc82Δ CPR7Δ cells in liquid medium but do not show increased β-galactosidase activity compared with wild-type cells. The level of HSF protein was found to be approximately equivalent in wild-type, hsc82Δ, CPR7Δ, and hsc82Δ CPR7Δ cells, Hsp90 and CPR7 do not appear to regulate the level of HSF protein, but rather may control the activity of HSF under normal conditions. Although β-galactosidase activity produced by pHSE2-lacZ is believed to reflect authentic HSF-mediated activity and has been widely used to study HSF regulation (32, 34, 35), we wished to authenticate the repressive effects of the Hsp90 machinery by monitoring the activity or abundance of proteins that are expressed from promoters harboring endogenous HSF-binding sites. In one set of experiments, a plasmid containing a fusion between the HSE2 element of the Hsp70-encoding gene SSA1 and CYC-lacZ (pZJHSE2–26 (33)) was introduced into wild-type and mutant cells and assayed for steady-state β-galactosidase activity. As observed in the experiments using the synthetic reporter gene, cells harboring mutations that decrease Hsp90 function exhibited increased β-galactosidase activity compared with wild-type cells (Fig. 1B). Furthermore, the extent of increase in reporter gene activity from hsc82Δ CPR7Δ cells compared with either single mutant indicated that Hsp90 and CPR7 act to synergistically repress HSF-dependent transcription (Fig. 1B).

Steady-state levels of Hsp104, a protein whose expression is regulated by HSF (15, 36) and is required for the acquisition of thermotolerance (36, 37), were also examined. Mutations that decrease Hsp90 activity were found to increase Hsp104 levels (Fig. 1C). Consistent with the reporter gene results, deletion of HSC82 conferred a modest increase in Hsp104, whereas deletion of both HSC82 and CPR7 resulted in dramatically higher steady-state levels of Hsp104 (Fig. 1C). Taken together, these results confirm that the transcriptional activity of HSF is negatively regulated by the Hsp90 machinery in vivo and establish CPR7 as an important component of this function.

To determine whether the Hsp90 machinery also regulates HSF activity during conditions that fully elicited the heat shock response, β-galactosidase activity produced by the pHSE2-lacZ reporter was measured in wild-type and Hsp90-mutant cells following a 30-min shift to 39 °C. Reporter gene activity in wild-type cells increased ~35-fold upon exposure to heat shock conditions (Fig. 2). Surprisingly, Hsp90-mutant cells exhibited even higher levels of β-galactosidase activity upon heat shock, with the greatest increase observed in hsc82Δ CPR7Δ cells (~98-fold compared with nonstressed wild-type cells) (Fig. 2). Thus, the Hsp90 machinery negatively regulates HSF activity even under conditions that elicit the full heat shock response.

Conditional Loss of Hsp90 Activity Confers Increased HSF-Dependent Gene Expression and Thermotolerance—If the Hsp90 machinery is required for repression of HSF activity under nonstress conditions, complete loss of Hsp90 function should result in increased HSF-mediated events under conditions that do not normally cause stress. It was not practical to test this prediction by deletion of both HSC82 and HSP82, because Hsp90 is essential for viability (28). Instead, we utilized a temperature sensitive allele of Hsp90 (hsp82G170D) that retains essentially wild-type activity at permissive temperatures (23 °C) but loses most of its activity at ~34 °C (30, 31). Thus, when the hsp82G170D allele is expressed in hsc82Δ hsp82Δ cells, Hsp90 activity can be controlled by altering the temperature at which cells are cultured (30, 31). Following a shift to 34 °C, these cells eventually stop dividing but remain viable for at least several hours (30, 31). Wild-type and hsc82Δ hsp82Δ hsp82G170D cells harboring the pHSE2-lacZ reporter gene were grown at 23 °C and either maintained at 23 °C or shifted to 34 °C and then assayed for β-galactosidase activity. Reporter gene expression in wild-type cells increased ~2.5-fold upon the 34 °C shift, reflecting a slight induction of the heat shock response at this temperature (Fig. 3A). In contrast, hsc82Δ hsp82Δ hsp82G170D cells displayed a ~30-fold increase in β-galactosidase activity (Fig. 3A). Thus, loss of Hsp90 function is sufficient to induce HSF-mediated gene expression.

A crucial aspect of activation of the heat shock response is the acquisition of a thermotolerant phenotype. We tested whether mutations in the Hsp90 machinery confer resistance to a sudden exposure to 52 °C for 4 min, a treatment that is normally lethal to cells that have not been allowed to mount a heat shock response. Although mutations that impair Hsp90 activity confer an increased steady-state level of Hsp104 (Fig. 1C), cells deleted for HSC82, CPR7, or both did not display a significant thermotolerant phenotype. This is consistent with suggestions that constitutively expressed Hsp104 is not as competent to protect cells from severe stress as newly synthesized protein (36). Therefore, we took advantage of the hsp82G170D allele to test whether a sudden loss of Hsp90 activity, which would be predicted to result in rapid synthesis of new Hsp104, confers thermotolerance. Wild-type and hsc82Δ hsp82Δ hsp82G170D cells cultured at 23 °C were either maintained at 23 °C or shifted to 33.5 or 37 °C and subsequently challenged with an incubation at 52 °C for 4 min. As expected, the survival rates of both cell types were very low (less than 0.01%) when cells were shifted directly from 23 °C to 52 °C but increased dramatically (up to 50%) if the cells were preincubated at 37 °C for either 1.5 or 3 h. Strikingly, hsc82Δ hsp82Δ hsp82G170D cells showed a 100–300-fold increase in thermotolerance compared with wild-type cells when the mutant cells were preincubated at 33.5 °C for 1.5 h (Fig. 3B) and 300–1,000-fold increase when the cells were preincubated at 33.5 °C for 3 h. This effect is not simply because the mutant cells stop dividing at elevated temperatures, because previous studies have shown that blockage of the cell cycle per se does not induce thermotolerance (38). To determine whether increased thermo-
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under both nonstress and stress conditions. The magnitude of increased expression of HSF-regulated genes in cpr7Δ cells showed that Cpr7 plays a major role in this regulation. Furthermore, our data demonstrate that Cpr7 and Hsp90 function to repress HSF-dependent gene expression in a synergistic manner, supporting the notion that the activities of Cpr7 and Hsp90 in regulation of the heat shock response are closely allied.

Expression of HSF-responsive genes increases sharply when cells are exposed to environmental stresses. We have discovered that in Hsp90 mutant cells heat shock regimens that are normally sufficient to confer the maximal heat shock response result in the induction of HSF-dependent genes to levels significantly greater than those observed in wild-type cells under the same conditions. This suggests that even during heat shock Hsp90 functions to negatively regulate HSF activity.

It is formally possible that increased expression of HSF-responsive genes in cells with reduced Hsp90 activity occurs through a mechanism independent of HSF. Because HSF is an essential gene in S. cerevisiae (10), this possibility cannot be tested directly by assessing the effects of Hsp90 mutations in cells lacking HSF. However, the observation that three different HSF-responsive genes are each affected in a similar manner by mutations in Hsp90 and CPR7 suggests that the increase in gene expression occurs via derepression of HSF activity. More importantly, the demonstration that an HSF-responsive reporter gene (HSE2::lacZ) that is made independent of HSF through point mutations that abolish HSF-binding (HSE2::lacZ) is no longer affected by Hsp90 mutations strongly supports the notion that Hsp90 and its associated proteins repress heat shock-related events by negatively regulating HSF activity.

How might the Hsp90 machinery regulate HSF activity? It is possible that negative regulation of HSF by Hsp90 occurs independently of the ability to associate with each other. For example, Hsp90 could separately interact with and inhibit a positive regulator of the heat shock response upstream from HSF. Alternatively, consistent with proposals that protein denaturation can signal activation of the heat shock response (4, 8, 9), one or more proteins might induce the response simply by failing to achieve native conformations in the absence of the chaperone. This would raise the intriguing possibility that disruption of the interaction between Hsp90 and certain substrates is a specific mechanism by which cells sense stress. Such a signal would likely be mediated by one or a few key factors and not by a state of general protein denaturation, because recent results demonstrate that the in vivo substrates of Hsp90 are highly restricted (30). Because Hsp90 can interact with HSF in both mammalian (18) and yeast cells (19), a particularly attractive model is one in which HSF is negatively regulated by physical association with Hsp90 under nonheat shock conditions. During heat shock, one or more components of the Hsp90 heterocomplex may dissociate from HSF in a temperature-dependent manner, thereby allowing HSF-mediated activation of transcription. Consistent with this proposal, Nair et al. (18) have shown that the Hsp90-HSF1 interaction can be disrupted by moderately elevated temperatures. Regulation of HSF activity by the Hsp90 complex is reminiscent of the role postulated for Hsp70 in HSF regulation (4, 8, 16). Whether Hsp70 and Hsp90 function independently in this regard is unknown. Nevertheless, the discovery that Hsp90 and a member of the CyP-40 class of cyclophilins are required for negative regulation of the heat shock response establishes the first specific endogenous role for these molecules in S. cerevisiae.

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