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Hsp90 Is Required for Pheromone Signaling in Yeast

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The heat-shock protein 90 (Hsp90) is a cytosolic molecular chaperone that is highly abundant even at normal temperature. Specific functions for Hsp90 have been proposed based on the characterization of its interactions with certain transcription factors and kinases including Raf in vertebrates and flies. We therefore decided to address the role of Hsp90 for MAP kinase pathways in the budding yeast, an organism amenable to both genetic and biochemical analyses. We found that both basal and induced activities of the pheromone-signaling pathway depend on Hsp90. Signaling is defective in strains expressing low levels or point mutants of yeast Hsp90 (Hsp82), or human Hsp90β instead of the wild-type protein. Ste11, a yeast equivalent of Raf, forms complexes with wild-type Hsp90 and depends on Hsp90 function for accumulation. For budding yeast, Ste11 represents the first identified endogenous "substrate" of Hsp90. Moreover, Hsp90 functions in steroid receptor and pheromone signaling can be genetically separated as the Hsp82 point mutant T525I and the human Hsp90β are specifically defective for the former and the latter, respectively. These findings further corroborate the view that molecular chaperones must also be considered as transient or stable components of signal transduction pathways.

INTRODUCTION

The 90-kDa heat-shock protein (Hsp90)[1] (for reviews, see Jakob and Buchner, 1994; Csermely *et al.*, 1998) is an ubiquitous and abundantly expressed cytosolic protein even at normal temperature. It is highly conserved from bacteria to mammals. Two genes encode closely related isoforms in mammals as well as in the budding yeast *Saccharomyces cerevisiae*. Deletion experiments in yeast have shown that the expression of at least one of the two Hsp90 isoforms, either Hsp82 or Hsc82, is essential for viability (Borkovich *et al.*, 1989). Similarly, many mutant alleles of the *Drosophila HSP90* homolog, *HSP83*, are embryonic lethals over a deficiency of the locus (van der Straten *et al.*, 1997), whereas the *Escherichia coli* homolog of Hsp90, HtpG, appears to be dispensable (Bardwell and Craig, 1988). Hsp90 can act as a molecular chaperone in vitro to promote refolding of denatured proteins (Wiech *et al.*, 1992; Yonehara *et al.*, 1996; see also Shaknovich *et al.*, 1992; Shue and Kohtz, 1994), to hold denatured proteins in a folding-competent state for other chaperones (Freeman and Morimoto, 1996; Yonehara *et al.*, 1996) and to prevent protein unfolding and aggregation (Miyata and Yahara, 1992; Jakob *et al.*, 1995a, 1995b; Yonehara *et al.*, 1996).

The interaction of Hsp90 with steroid receptors, which can be thought of as a signal transduction complex, has been the most extensively investigated. A variety of in vitro and in vivo studies have revealed that steroid receptors are complexed with Hsp90 and several other proteins in the absence of hormone (for review, see Pratt and Toft, 1997). Upon ligand binding, the hormone binding domain (HBD) undergoes a conformational change that results in the release of Hsp90 and the concomitant activation of the steroid receptor. Steroid receptors and many heterologous proteins fused to the HBD are maintained inactive in the absence of hormone. We have therefore hypothesized that the hormone-reversible inactivation function of the HBD is mediated by Hsp90, possibly by steric hindrance (Picard, 1993, 1994). Further insights into

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[1] Abbreviations used: HBD, hormone-binding domain; Hsp, heat-shock protein.

the role of Hsp90 in the regulation of this particular signal transduction pathway come from studies made in yeast (reviewed in Picard, 1998). Vertebrate steroid receptors expressed in yeast strains with a low level (Picard *et al.*, 1990; see also Holley and Yamamoto, 1995) or specific point mutants of Hsp82 (Bohen and Yamamoto, 1993; Bohen, 1995; Nathan and Lindquist, 1995; Fang *et al.*, 1996) show a defective hormonal response that is due to a decrease in the ligand-binding affinity (Bohen, 1995; Fang *et al.*, 1996). Thus, Hsp90 may have a dual role: it ensures that receptors are kept inactive in the absence of hormone and helps them to respond specifically and efficiently to ligand. This view is also corroborated by pharmacological in vivo experiments with geldanamycin (Whitesell *et al.*, 1994), a compound that interferes with certain Hsp90 functions such as the proper maturation of steroid receptor–Hsp90 complexes (Smith *et al.*, 1995; Whitesell and Cook, 1996; Bamberger *et al.*, 1997; Czar *et al.*, 1997; Segnitz and Gehring, 1997).

There is ample evidence for a role of Hsp90 in regulating the activity of several other signaling pathways, such as the xenobiotic response mediated by the dioxin receptor (see for example Pongratz *et al.*, 1992; Carver *et al.*, 1994; McGuire *et al.*, 1994; Antonsson *et al.*, 1995; Coumailleau *et al.*, 1995; Whitelaw *et al.*, 1995). Interaction of the dioxin receptor with Hsp90 is essential for ligand binding and for acquiring a DNA-binding conformation. Activation of the dioxin receptor depends on the release of Hsp90 upon ligand binding and heterodimerization with Arnt. A functional dependence on, and a direct interaction with, Hsp90 has also been described for kinases such as the fission yeast Wee1 (Aligue *et al.*, 1994), the vertebrate v-Src (Schuh *et al.*, 1985; Xu and Lindquist, 1993; Nathan and Lindquist, 1995), and the related kinase Lck (Hartson *et al.*, 1996).

Hsp90 may also be required for growth factor signaling. 1) Raf-1, a serine/threonine kinase involved in mitogenic signal transduction in vertebrates, exists in a geldanamycin-sensitive heterocomplex with Hsp90 (Stancato *et al.*, 1993, 1994; Lovric *et al.*, 1994; Wartmann and Davis, 1994; Schulte *et al.*, 1995, 1996; Stancato *et al.*, 1997). 2) Mutations in *Drosophila HSP83* reduce signaling by the torso (Doyle and Bishop, 1993) and sevenless receptors (Cutforth and Rubin, 1994; van der Straten *et al.*, 1997), which may be due, at least in part, to a requirement for Hsp90 for Raf function (van der Straten *et al.*, 1997). 3) The insulin receptor binds Hsp90, and antibodies to Hsp90 interfere with insulin signaling (Takata *et al.*, 1997).

Comparable MAPK pathways also exist in yeast where they regulate the pheromone response, invasive growth, pseudohyphal development, osmoregulation, cell wall integrity, and sporulation (for reviews, see Herskowitz, 1995; Levin and Errede, 1995; Schultz *et al.*, 1995; Leberer *et al.*, 1997). The pheromone-signaling pathway has received a lot of attention over the past few years. Binding of the mating pheromones to transmembrane receptors elicits a series of events including the sequential activation of the kinases Ste11, Ste7, and Fus3, leading to morphological changes, a cell cycle arrest in G1, and the expression of specific genes required for mating. The kinase Ste11 from *S. cerevisiae* occupies a position analogous to that of Raf. This prompted us to test genetically whether Hsp90 plays a role in the pheromone pathway.

MATERIALS AND METHODS

Plasmids

Hsp90 plasmids. Wild-type Hsp82 (Hsp82 wt), Hsp82 G313N, and Hsp82 T525I were expressed from plasmids pTCA/Hsp82, pTCA/Hsp82 G313N, and pTCA/Hsp82 T525I, respectively (Bohen, 1995), or various derivatives thereof with other auxotrophic markers. Unless indicated, the strong constitutive promoter from the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene *TDH3* was used to drive expression. Plasmid pHCA/Hsp82 is the *HIS3* version of pTCA/Hsp82 obtained by substituting the backbone of shuttle vector pRS313 for that of pRS314 (Sikorski and Hieter, 1989). Plasmid p2U/Hsp82, a 2μ-*URA3* expression vector for Hsp82 has been described previously (Louvion *et al.*, 1996).

To obtain reduced levels of Hsp82 (~10% of Hsp82 + Hsc82 in a wild-type strain), Hsp82 was expressed from a construct containing the leaky *GAL1* promoter from strain GRS4 (Picard *et al.*, 1990) fused to *HSP82* coding sequences in plasmid pRS304 (Sikorski and Hieter, 1989). On medium with 2% glucose, repression of this mutant *GAL1* promoter construct is only partial, and low levels of Hsp82 accumulate.

Plasmid p2TG/hHsp90β expressing human Hsp90β was constructed as follows. The coding sequence for human Hsp90β was excised as a *Sna*BI–*Sal*I fragment from pKN1–3 (Rebbe *et al.*, 1987) and cloned into the *Sma*I site of pSP64 to add a *Bam*HI site at the 5′-end and a *Sac*I site at the 3′-end. The *Bam*HI–*Sac*I fragment containing the human *HSP90β* sequence was fused to the GPD promoter in shuttle vector pRS304 (Sikorski and Hieter, 1989) with a 2μ replicon. Plasmid p2HG/hHsp90β is the *HIS3* version based on expression vector p2HG (Picard *et al.*, 1990).

Plasmids p2G/Hsp82, p2G/Hsp82 G313N, and p2G/hHsp90β are identical to plasmids p2HG/Hsp82 (Louvion *et al.*, 1996), p2HG/Hsp82 G313N (the G313N derivative of p2HG/Hsp82), and p2HG/hHsp90β, respectively, except that they lack an internal *Hin*dIII fragment of the *HIS3* marker. Thus, rather than an auxotrophic marker it is the Hsp90 function itself that provides the selectable marker for these plasmids.

Plasmid p2TG/flag.Hsp82wt serves to express Hsp82 with a FLAG epitope at the N terminus. The expression vector was derived from p2TG/hHsp90β. Sequences encoding the FLAG epitope (DYKDDDDK) were placed between the initiator codon and the second codon of the wild-type *HSP82* sequences, following the introduction of a *Bgl*II site just upstream of the second nucleotide of the *HSP82*-coding sequence. FLAG epitope and second amino acid of Hsp82 are thus separated by the three extra amino acids EIL.

Other Plasmids. Plasmid pYES/Ste11ΔN encoding Ste11ΔN was generated as follows: the coding sequence for the catalytic domain of Ste11 was excised from plasmid pNC199 (a gift from B. Errede) as a *Dde*I–*Bgl*II fragment and subcloned into pSP72 to add a *Bam*HI site at the 5′-end. This fragment was further subcloned as a *Bam*HI–*Bgl*II fragment into a pUC18 derivative containing a stop codon in the proper reading frame followed by a *Sac*I site. Finally, the sequence encoding the catalytic domain of Ste11 was introduced into plasmid pYES 2.0 (Invitrogen, San Diego, CA) as a *Bam*HI–*Sac*I fragment. pYES 2.0 is a yeast expression vector that contains the galactose-

inducible *GAL1* promoter, the 2μ replicon, and the *URA3* selectable marker. Plasmid pYES/HA-Ste11 was constructed for galactose-inducible expression of full-length Ste11 with an influenza virus hemagglutinin (HA) epitope (Daro *et al.*, 1996) at its N terminus; a *Kpn*I–*Nde*I fragment with sequences encoding the HA epitope (MQDLPGNDNSTAG) was joined in-frame to a *Bam*HI fragment carrying *STE11*-coding sequences from plasmid BB345 (mentioned as pYBS345 in Choi *et al.*, 1994) and cloned into pYES 2.0 linearized with *Kpn*I and *Not*I; noncomplementary sites were filled in or chewed back to allow ligation.

Plasmid pUCA/Ste7 M was used to express a myc-tagged Ste7 protein. It contains the *CYC1* promoter and Ste7-coding sequences from plasmid pNC318 (Zhou *et al.*, 1993) excised as a *Sal*I–*Hin*dIII fragment and cloned into the *Sal*I–*Sma*I linearized plasmid pRS316 (Sikorski and Hieter, 1989).

The yeast genomic library (a gift obtained via M. Collart) was a *Sau* 3A partial library cloned into the *Bam*HI site of the 2μ-*URA3* vector YEplac195 (Gietz and Sugino, 1988).

Plasmids p2U/GST-2 (Warth *et al.*, 1997), p2U/GST-STE5, and pYes/Ste11ΔN.GST served to express glutathione-*S*-transferase (GST), GST fused to Ste5, and GST fused to Ste11ΔN, respectively. p2U/GST-STE5 was constructed by replacing the *Bam*HI–*Bgl*II fragment at the 5′-end of *HSP82* of p2U/Hsp82 with a *Bam*HI fragment carrying GST-coding sequences fused in-frame to *STE5* sequences; *STE5* sequences lacking the first 24 codons were from plasmid BB192 (mentioned as pYBS146 in Choi *et al.*, 1994).

Strains

The parent strains and some of the derivatives are listed in Table 2. The related yeast strain backgrounds, HH1a and JC6a (gifts from S. Lindquist), were used to replace the endogenous Hsp82/Hsc82 with Hsp90 mutants by plasmid shuffling. Plasmids were introduced into yeast by the LiAc/PEG method and selected for on appropriate minimal media. Strain HH1a-pHCA/Hsp82wt is essentially the *MAT*a version of the previously described strain HH1-KAT6 (see Palmer *et al.*, 1995). It was obtained by tetrad dissection of a diploidized HH1-KAT6 and further plasmid shuffling.

The strain DP121 was obtained by substituting the *HIS3* coding body for that of *FUS1* in strain DP120 (see Table 2) with the gene replacement construct pSL1497 (Stevenson *et al.*, 1992). Plasmid p2U/Hsp82 was subsequently replaced by the Hsp90 expression vectors pTCA/Hsp82, pTCA/Hsp82 G313N, and p2TG/hHsp90β, to yield strains DP122, DP123, and DP124, respectively. In strains HH1a-p2G/Hsp82wt, HH1a-p2G/Hsp82 G313N, and HH1a-p2G/hHsp90, the Hsp90 derivatives themselves are used as selectable marker to maintain the episomes.

α-Factor Induction

To monitor the cell cycle arrest in response to α-factor, cells were diluted to a density of 1.2×10^7 cells/ml and streaked or spotted onto YEPD plates containing 10 mM Na-citrate, pH 4.3, and, where indicated, 5 μM α-factor (Bachem, Torrance, CA). The *FUS1-LacZ* reporter plasmid pSB234 was used to measure the transcriptional output of the pheromone pathway (Trueheart *et al.*, 1987). Wild-type and mutant strains were grown to early logarithmic phase and exposed to 5 μM α-factor for 2 h after addition of 10 mM Na-citrate, pH 4.3. Quantification of the LacZ expression was performed as described by Yocum *et al.* (1984) except that chlorophenol red-β-D-galactopyranoside was used as β-galactosidase substrate instead of *O*-nitrophenyl β-D-galactopyranoside for more sensitivity.

Rapid Protein Extraction

The levels of overexpressed Ste11 (yeast strains JC6a-Hsp82, JC6a-Hsp82 G313N, and JC6a-hHsp90β with plasmid pYES/HA-Ste11) were quantitated using crude extracts prepared by a rapid protein extraction protocol (Horvath and Riezman, 1994) and loaded onto 10% SDS-polyacrylamide gels. To confirm that equal amounts of protein had been loaded, proteins were stained with Ponceau S after transfer onto a nitrocellulose membrane before immunostaining.

Analysis of Ste7 Phosphorylation

JC6a strains expressing the Hsp90 derivatives were transformed with plasmid pUCA/Ste7 M. Transformants were grown to early logarithmic phase in 1% sucrose as a carbon source. After addition of 10 mM Na-citrate, pH 4.3, the cultures were exposed to 5 μM α-factor for 2 h. Cell extracts were prepared at 4°C by breaking the cells with glass beads in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 20 mM sodium molybdate, 15 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, the protease inhibitors aprotinin, leupeptin, and pepstatin A, and the phosphatase inhibitors okadaic acid (1 μM), Na_2MoO_4 (10 mM), Na_3VO_4 (0.1 mM), and NaF (5 mM). Samples were frozen in liquid nitrogen and stored at −70°C. Extracts, 10 μg each, as determined with the Bio-Rad (Richmond, CA) Bradford reagent, were boiled in SDS sample buffer for 5 min and loaded onto 7.5% SDS-polyacrylamide gels.

GST Pull-Down and Immunoprecipitation Experiments

GST pull-down experiments were performed as follows. Yeast cells (strain RMY326 with plasmids pYes/Ste11ΔN.GST or p2U/GST-2) were washed once with water containing 1 mM DTT and 1 mM PMSF and once with TEG (25 mM Tris-HCl pH 7.4, 15 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 3 μg/ml chymostatin, 1.5 μg/ml pepstatin A, 0.75 μg/ml leupeptin, 3.8 μg/ml antipain) containing 150 mM NaCl. Cell pellets were then resuspended in a small volume of the same buffer and broken with glass beads by two 30-s pulses at maximum speed in a Mini-BeadBeater-8 (Biospec Products, Bartlesville, OK) at 4°C. After centrifugation at 15,000 rpm in a table top centrifuge at 4°C, the supernatant was quantitated and adjusted to 0.1% Triton X-100. Glutathione-sepharose beads (Pharmacia, Piscataway, NJ) were added to the extracts, tumbled for 30–45 min at 4°C, washed three times with TEG containing 150 mM NaCl, 0.1% Triton X-100 and twice with TEG with 0.1% Triton X-100. Bound proteins were eluted with 7.5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and concentrated by trichloroacetic acid (TCA) precipitation, resuspended in SDS sample buffer, and loaded onto 10% SDS-polyacrylamide gels.

Coimmunoprecipitation experiments using the FLAG tag were done as follows. Extracts from strains HH1a-p2TG/flag.Hsp82wt and HH1a-p2G/hsp82wt with and without plasmid pYES/HA-Ste11 were prepared as described above for the GST pull-down experiments except that the buffer was 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10 mM sodium molybdate, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 3 μg/ml chymostatin, 1.5 μg/ml pepstatin A, 0.75 μg/ml leupeptin, 3.8 μg/ml antipain. After adjusting the extracts to 0.1% Triton X-100, they were incubated at 4°C with the anti-FLAG monoclonal antibody M2 (Eastman Kodak, Rochester, NY) for 2 h followed by 1 h with Protein G-sepharose (Pharmacia). Immunoprecipitates were washed four times for 10 min at 4°C with the extraction buffer containing 0.1% Triton X-100, solubilized in SDS sample buffer, and loaded onto 10% SDS-polyacrylamide gels. The same protocol was used for immunoprecipitation by a Ste11-specific rabbit polyclonal antiserum (Cairns *et al.*, 1992) of endogenous Ste11 from 0.5 mg of extracts from strains HH1a-p2G/Hsp82wt, HH1a-p2G/Hsp82 G313N, and HH1a-p2G/hHsp90β.

Western Blot Experiments

After transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membranes, the membranes were blocked with Tris-buffered saline, 0.05% Tween-20 (TBST) containing 5% (wt/vol) milk powder and probed with appropriate antibodies in TBST + milk powder at room temperature for 1 h. Mouse anti-GST (Santa Cruz Biotechnol-

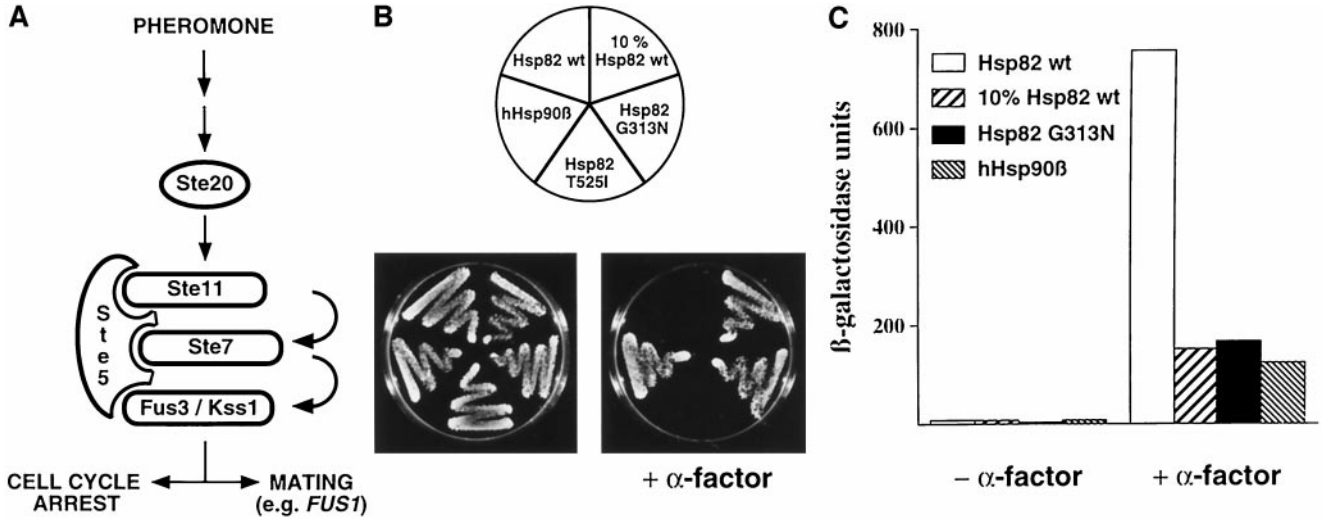

Figure 1. Hsp90 is required for pheromone signaling. (A) Schematic representation of the pheromone pathway of budding yeast. Protein kinases Ste11, Ste7, and Fus3/Kss1 are the yeast equivalents of MEKK, MEK, and MAPK, respectively, and are held together by Ste5. Pheromone signaling results in the activation of Ste12 and Far1. The transcription factor Ste12 controls the expression of mating genes, whereas Far1 inhibits G_1 cyclins and thereby effects a cell cycle arrest. (B) Yeast strains with *HSP90* mutations fail to respond to α-factor. Strains expressing the indicated Hsp90 derivatives were streaked onto YEPD plates with or without 5 μM α-factor. The plates were photographed after 3 d of incubation at 30°C. (C) Induction of the *FUS1-LacZ* reporter gene by α-factor is strongly reduced in strains with *HSP90* mutations. Strains expressing the indicated Hsp90 derivatives were tranformed with the *FUS1-LacZ* reporter construct. β-Galactosidase expression was quantitated after exposure to α-factor for 2 h.

ogy, Santa Cruz, CA), anti-HA (a gift from K. Matter; for references, see Daro *et al.*, 1996), and anti-FLAG (Kodak) monoclonal antibodies, chicken anti-Hsp82 antibodies (Louvion *et al.*, 1996), rabbit polyclonal anti-Hsp82 antiserum (a gift from S. Lindquist), and rabbit polyclonal anti-Ste11 antiserum (Cairns *et al.*, 1992) were diluted 1:1000, 1:100, and to 10 μg/ml, 1:1000, 1:400, and 1:1000, respectively. Membranes were washed three times for 10 min with TBST. The secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit (Bio-Rad) or anti-chicken (Promega, Madison, WI), horseradish peroxidase-conjugated anti-mouse (Cappel, Cochranville, PA). They were used in TBST + milk powder at room temperature for 1 h. After three washes with TBST, the blots were developed either with the NBT/BCIP reagent for alkaline phosphatase or with the enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL) for horseradish peroxidase.

RESULTS

HSP90 Mutations Interfere with Pheromone-induced Cell Cycle Arrest and Activation of FUS1 Promoter

A large variety of *HSP90* mutants have been described that complement yeast strains carrying disruptions of the essential chromosomal *HSP90* genes, *HSP82* and *HSC82* (Borkovich *et al.*, 1989; Picard *et al.*, 1990; Bohen and Yamamoto, 1993; Kimura *et al.*, 1994; Minami *et al.*, 1994; Bohen, 1995; Nathan and Lindquist, 1995; Palmer *et al.*, 1995; Louvion *et al.*, 1996). We examined pheromone signaling (Figure 1A) in three types of mutant strains: a strain with only 10% of the normal levels of Hsp82, a strain with human Hsp90β (hHsp90β; hereafter considered a Hsp90 mutant for yeast), and strains expressing specific Hsp82 point mutants. The latter had been found in a screen for defective steroid receptor signaling in yeast. The point mutants T525I and G313N are temperature sensitive for viability and show an impaired hormonal response of glucocorticoid, estrogen, progesterone, and mineralocorticoid receptors (Bohen and Yamamoto, 1993; Bohen, 1995). Hsp82 T525I and Hsp82 G313N are expressed at similar levels as the wild-type Hsp82 (Bohen and Yamamoto, 1993; Bohen, 1995) (see also Figure 4B). We first tested the different mutant strains for their ability to arrest growth in response to the mating pheromone α-factor. As shown in Figure 1B, low levels of Hsp82, point mutant Hsp82 G313N, and hHsp90β are not able to promote a substantial activation of the pheromone pathway as demonstrated by a poor growth arrest in the presence of pheromone. Interestingly, the point mutation T525I discriminates between two different functions of Hsp90, the pheromone and the steroid-signaling pathways being functional and defective, respectively. The other Hsp90 isoform of yeast, Hsc82, as well as the *Trypanosoma cruzi* Hsp83, which we have previously shown to complement defective yeast strains (Palmer *et al.*, 1995), are also able to support pheromone signaling (our unpublished results).

The activation of the pheromone pathway also results in the induction of proteins required for cellular and nuclear fusion (mating). To test the Hsp90 requirement in the pheromone-dependent transactiva-

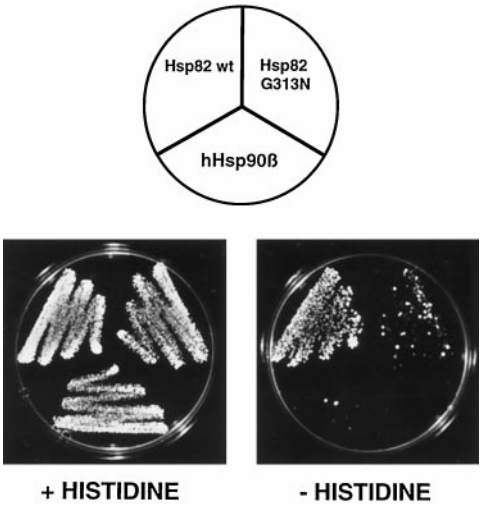

Figure 2. The basal activity of the pheromone-signaling pathway depends on Hsp90. Strains expressing wild-type (DP122) or mutant Hsp90 (DP123 and DP124) and carrying the *FUS1-HIS3* reporter gene were grown on indicator plates containing or lacking histidine. The strains are all derivatives of strain DP121. The plates were photographed after 3 d of incubation at 30°C.

tion of mating genes such as *FUS1*, we assessed the induction of a *FUS1-LacZ* reporter gene (Trueheart *et al.*, 1987) upon treatment of cells with α-factor. The results shown in Figure 1C indicate that, similarly to what was observed in the case of the G_1 arrest, the activation of *FUS1-LacZ* in response to α-factor is strongly reduced in Hsp90 mutant strains compared with a strain with wild-type Hsp82.

Basal Activity of the Pheromone-signaling Pathway Also Depends on Hsp90

The pheromone pathway exhibits low activity even in the absence of pheromones (Hagen *et al.*, 1991). To determine whether Hsp90 is also required for this basal activity, we examined the activity of a more sensitive reporter gene, *FUS1-HIS3*, in a *his3*$^-$ strain. Any disruption in the pheromone signaling pathway, such as the complete absence of a component, abrogates the basal activity and prevents growth on medium lacking histidine (Stevenson *et al.*, 1992). As shown in Figure 2, growth on selective medium is severely impaired for Hsp82 G313N and hHsp90β strains when compared with a strain with wild-type Hsp82. In this assay, the hHsp90β strain is reproducibly the most defective. These results indicate that Hsp90 is necessary for both the induced and the basal activity of the pheromone-signaling pathway.

Hsp90 Mutants Block Signaling by Constitutive Ste11

In a first attempt toward determining the step(s) of the pheromone pathway (Figure 1A) that is dependent on Hsp90, we assayed growth arrest induced by a constitutively active Ste11 mutant. It has been shown that the deletion of the amino-terminal regulatory domain of Ste11 (Ste11ΔN) results in constitutive activation of this kinase and in pheromone-independent induction of the mating pathway (Cairns *et al.*, 1992). We constructed such a dominant *STE11* and placed it under the control of the conditional *GAL1* promoter. When the expression is induced by growth on galactose, only the strain with wild-type Hsp82 exhibits a complete growth arrest (Figure 3A). Strains with either Hsp82 G313N or hHsp90β fail to be fully growth arrested. The same pattern was observed with equivalent strains of the opposite mating type (*MAT*α) (our unpublished results). Thus, these experiments showed that the requirement for Hsp90 is independent of mating type and possibly at the level of Ste11 or downstream of it.

Ste5 Overexpression Suppresses the Signaling Defect

We performed a screen for high-copy suppressors of the signaling defect of Hsp90 mutant strains. Using the hHsp90β strain with the *FUS1-HIS3* reporter, we selected suppressors that allow growth on plates lacking histidine in the absence of pheromone and screened them further for a restored sensitivity to α-factor. In a limited screen with a yeast genomic library in a high-copy vector, only one clone met the two criteria. Sequencing revealed that its genomic insert contains the *STE5* gene. It starts 1020 bp upstream of the initiator codon ATG and presumably contains the complete *STE5* promoter. At the 3′-end the *STE5* sequence is truncated at codon 801 (of 917). The isolated plasmid, denoted Ste5ΔC, thus encodes the first 800 amino acids of Ste5 fused to 15 unrelated amino acids at the C terminus. Further experiments showed that Ste5ΔC is also able to suppress the *HSP82* mutation G313N. Figure 3B shows the growth assays on plates lacking histidine and also illustrates that full-length Ste5, as a fusion protein with GST, retains suppressor activity. These data corroborate the tentative conclusion that Hsp90 may be required at the level of the MAPK module consisting of the kinases Ste11, Ste7, and Fus3 that are tethered together by Ste5 (reviewed by Elion, 1995; Leberer *et al.*, 1997).

Ste11 Protein Levels Are Reduced

Certain client proteins of Hsp90, such as Raf-1, the glucocorticoid receptor, or luciferase, appear more susceptible to degradation when interaction with Hsp90 is blocked/altered pharmacologically (Schulte *et al.*, 1995–1997; Schneider *et al.*, 1996; Whitesell and Cook, 1996; Czar *et al.*, 1997; Segnitz and Gehring, 1997; Stancato *et al.*, 1997). We therefore examined the accumulation of Ste11 in Hsp90 mutant strains. An epitope-tagged version of Ste11 was overexpressed

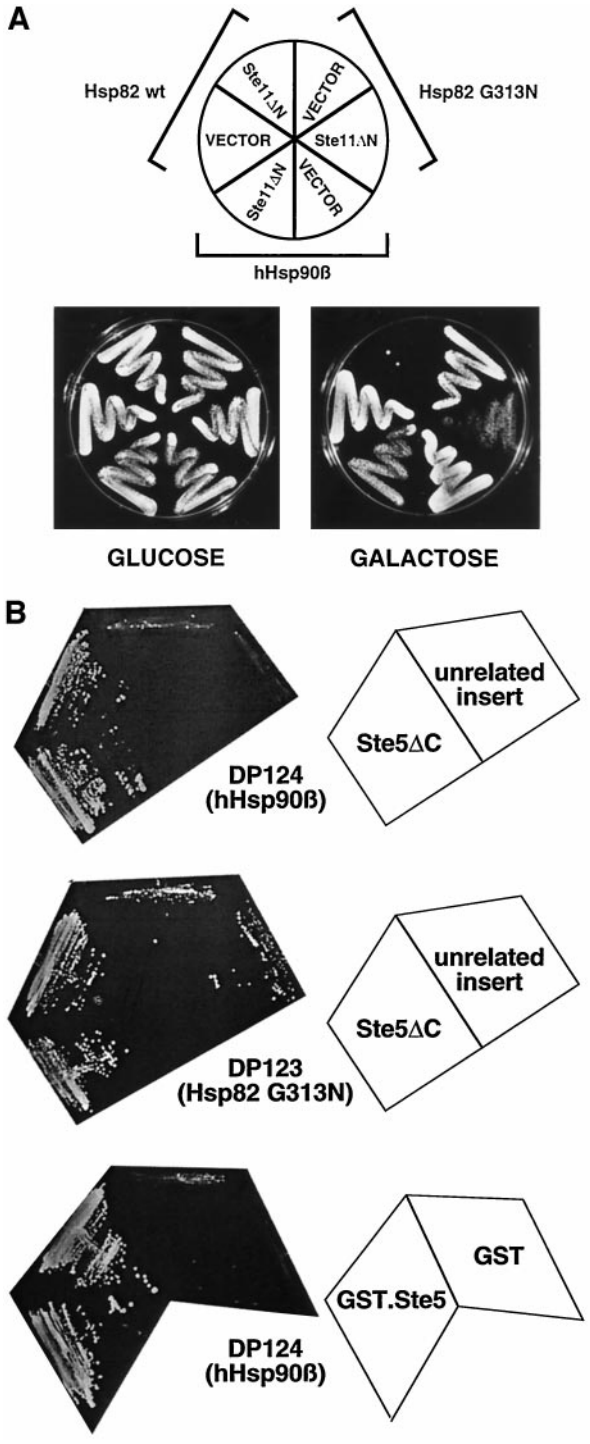

Figure 3. Hsp90 is required for signaling beyond Ste11 at the level of the MAPK module. (A) Signaling by the constitutively active Ste11ΔN mutant is blocked by *HSP90* mutations. Ste11ΔN is conditionally expressed under the control of the *GAL1* promoter. Transformants containing the expression plasmid encoding Ste11ΔN (pYES/Ste11ΔN) or the empty plasmid pYES 2.0 (VECTOR) were precultured in selective medium containing glucose as a carbon source and streaked onto repressing (glucose) and inducing (galactose) plates and incubated for 4 d. (B) Ste5 suppresses the signaling defect of Hsp90 mutants. Plasmids encoding C-terminally–truncated Ste5 (Ste5ΔC) and GST fused to full-length Ste5 (GST.Ste5) were introduced into strains carrying the *FUS1-HIS3* reporter gene and expressing hHsp90β (strain DP124) or the Hsp82 mutant G313N (strain DP123). The negative controls for Ste5ΔC and GST.Ste5 were another clone from the same library with an unrelated insert and a plasmid expressing GST alone, respectively. Two colonies each were streaked out on indicator plates lacking histidine. The plates were photographed after 3 d of incubation at 30°C.

under the control of the inducible *GAL1* promoter and revealed by immunoblotting (Figure 4A, left panel). In strains with hHsp90β or Hsp82 G313N, Ste11 levels were severely reduced. In the Hsp82 G313N strain Ste11 levels were at the detection limit. At this point we speculated that the levels of the endogenous Ste11, which is difficult to detect, might mirror this pattern. To explore this possibility, we concentrated endogenous wild-type Ste11 by immunoprecipitation with a Ste11-specific antiserum and displayed it by immunoblotting with the same antiserum (Figure 4A, right panel). Despite a relatively high background, the identity of the Ste11 band could be confirmed unambiguously using an extract from a *ste11*$^{-}$ strain as a control sample (Figure 4A, lane Δ). As in the case of the overexpressed Ste11, accumulation of endogenous Ste11 is reduced in both mutant strains although Hsp82 G313N appears to have a less severe effect on the endogenous than on the overexpressed protein. Thus, reduced levels of Ste11 could, at least in part, explain the functional defects of the pheromone pathway in these strains.

Basal and Induced Phosphorylation of Ste7 Is Reduced

The direct target of the Ste11 kinase is the kinase Ste7 (Figure 1A). Upon exposure to pheromone, Ste7 is activated by phosphorylation by Ste11 and becomes hyperphosphorylated in the presence of Fus3/Kss1 (Zhou *et al.*, 1993; Neiman and Herskowitz, 1994). As shown in Figure 4B, the degree of hyperphosphorylation of Ste7 as well as Ste7 protein levels is strongly reduced in mutant strains. The latter is particularly true for Hsp82 G313N. When compared with the wild-type strain, all the mutant strains show a three- to fivefold reduced hyperphosphorylation of Ste7 both in the absence (basal activity) and in the presence (induced activity) of pheromone. This experiment indicates that Hsp90 function is essential both for Ste7 accumulation and for efficient basal and induced phosphorylation of Ste7.

Ste11 Forms Complexes with Hsp90

Several experiments described so far suggested that Hsp90 might interact with components of the MAPK

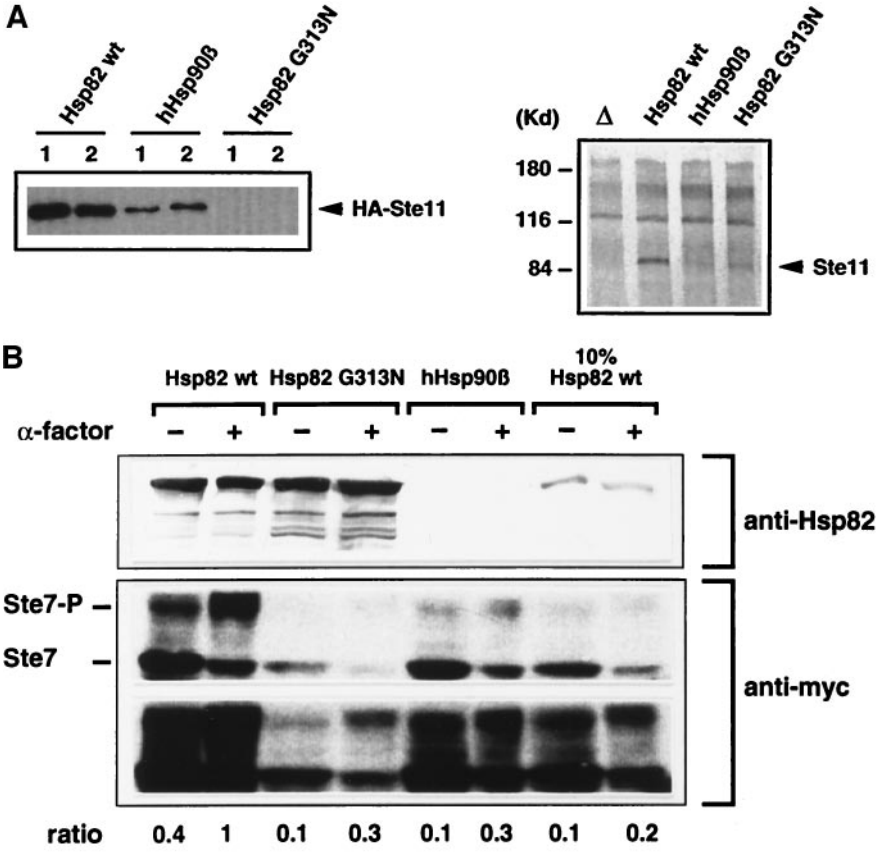

Figure 4. HSP90 mutations affect the levels of Ste11 and Ste7, and Ste7 hyperphosphorylation. (A) Ste11 protein levels are reduced in Hsp90 mutant strains. Left panel, HA-epitope–tagged Ste11 was overexpressed in Hsp90 mutant strains as indicated; equal amounts of "rapid protein extracts" of two colonies each (1 and 2) were immunoblotted with anti-HA antibodies. Right panel, endogenous wild-type Ste11 was concentrated by immunoprecipitation and immunoblotted with a Ste11-specific antiserum; lane Δ, extracts from a Δ*ste11* control strain. (B) Phosphorylation of Ste7 is reduced by *HSP90* mutations. Strains expressing Ste7 containing the myc epitope were exposed for 2 h to α-factor where indicated. Extracts were analyzed by an immunoblot assay using a rabbit polyclonal antibody (a gift from S. Lindquist), which only recognizes the yeast Hsp82 (top panel), and the mouse monoclonal antibody 9E10 against the myc epitope (bottom panel). Two different exposures of the latter immunoblot are presented. The position of the hyperphosphorylated Ste7 (Ste7-P) is indicated. The ratios of hyperphosphorylated Ste7 (Ste7-P) over unphosphorylated Ste7 of all samples were standardized on the ratio obtained in the presence of α-factor with the strain expressing wild-type Hsp82.

module and Ste11 in particular. We performed coprecipitation experiments to examine this issue. Figure 5A shows that HA epitope-tagged Ste11 is specifically coprecipitated with FLAG-tagged Hsp82. The association of Ste11 and yeast Hsp90 (Hsp82) was confirmed by a GST pull-down experiment. GST alone or GST fused to the constitutive Ste11ΔN (Ste11ΔN.GST) was inducibly expressed under the *GAL1* promoter in a wild-type strain. While wild-type Hsp82 (and Hsc82) does not associate with GST alone, it specifically coprecipitates with Ste11ΔN.GST (Figure 5B). These results establish that Ste11 exists in complexes with Hsp90 and that the regulatory N-terminal domain of Ste11 is dispensable for this interaction.

Differential Temperature Sensitivity of Hsp90 Mutants

Surprisingly, the Hsp82 requirement for pheromone signaling exhibits a temperature dependence. The mutant phenotype of Hsp82 G313N and hHsp90β strains was not apparent in all assays at the lower temperature of 21°C (Table 1). This is particularly striking for G313N whose response in all our assays is only slightly reduced compared with wild-type Hsp82 at 21°C. In contrast, low amounts of wild-type Hsp82 are unable to support signaling in response to α-factor even at the lower temperature. Similarly, the basal and α-factor–induced activities of the pheromone pathway are defective in strains with hHsp90β at both temperatures. Interestingly, a full growth arrest is observed at 21°C with a hHsp90β strain when the pheromone pathway is activated with the constitutive Ste11ΔN. This suggests that Hsp90 function might be required differentially both "upstream" and "downstream" of Ste11. At low temperature, hHsp90β appears to be able to fulfill the downstream, but not the upstream requirement. Since none of the mutations are able to block the Ste11ΔN-induced cell cycle arrest at the lower temperature, we cannot formally rule out the possibility that Hsp90 function is not required at all for this particular response. However, this seems unlikely in view of the striking signaling defects at 30°C and may be due to the vast overexpression of Ste11ΔN in this assay and initiation of signaling at an intermediate level.

DISCUSSION

Using a series of Hsp90 mutants we have demonstrated that pheromone signaling through the MAPK cascade depends on Hsp90 function. Hsp90 is required both for the basal activity of this pathway in

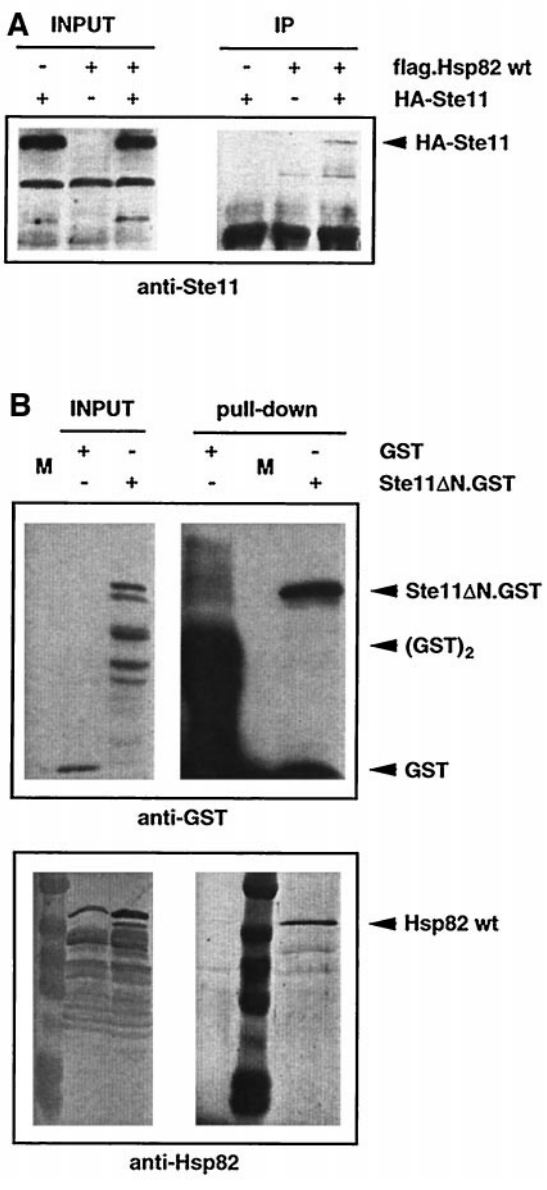

Figure 5. Ste11 forms complexes with Hsp90. (A) Immunoprecipitation experiment showing association of HA-tagged Ste11 with FLAG-tagged Hsp82. INPUT and IP designate equal amounts of total extract and immunoprecipitates, respectively (exposure times are not the same). Note that isogenic strains were used expressing Hsp82 with or without the FLAG tag. HA-Ste11 was revealed by immunoblotting with an anti-Ste11 antiserum. (B) GST pull-down experiment showing association of Ste11ΔN.GST with endogenous Hsp90 (Hsp82/Hsc82). INPUT and "Pull-down" designate equal amounts of total extract and proteins purified with glutathione-sepharose beads, respectively (exposure times are not the same). GST and Ste11ΔN.GST and Hsp82/Hsc82 were revealed by probing the same blot with anti-GST and chicken anti-Hsp82 antibodies, respectively. Note that unfused GST binds the affinity matrix more efficiently, resulting in very strong GST monomer and dimer bands.

the absence of pheromone and for efficient induction upon exposure to pheromone. A combination of genetic and biochemical experiments pinpoints Ste11, a yeast equivalent of Raf, as a target of Hsp90. Since mammalian Raf-1 can substitute for Ste11 under certain circumstances (Freed *et al.*, 1994; Irie *et al.*, 1994), our results also set the stage for using yeast genetics to investigate the role of Hsp90 for Raf function and for mammalian MAPK signaling.

Ste11 Depends on Hsp90 Function

Our results support the conclusion that pheromone signaling depends on Hsp90 at the level of Ste11: 1) The constitutive Ste11 mutant (Ste11ΔN) fails to elicit a complete cell cycle arrest (at 30°C) in Hsp90 mutant strains; 2) The levels of both endogenous and overexpressed Ste11 are reduced in mutant strains; 3) The basal and induced phosphorylation of the Ste11 substrate Ste7 are reduced in mutant strains; 4) Ste11 and Hsp90 (Hsp82) are found in a complex.

The reduction of Ste11 protein levels are an indication that Hsp90 may be required to ensure the stability of Ste11. Since the plasmids that we used for overexpression of Ste11 contained exclusively the *STE11* coding body, the rate of synthesis is likely to be similar. This leads to the tentative conclusion that it is the turnover of Ste11 that is increased in Hsp90 mutant strains. Whether the destabilization of Ste11 is due to misfolding and/or a failure to form complexes with other factors remains to be determined. While the effects on Ste11 protein levels could also be indirect, the finding that Hsp90 and Ste11 form complexes suggests that it is the altered nature of these complexes in Hsp90 mutant strains that leads to enhanced degradation of Ste11. The low levels of Ste11 in these strains have so far precluded experiments to determine whether Hsp90 mutants form complexes with Ste11 at all. In vitro experiments with purified components might allow assessment of whether the Ste11-Hsp90 interaction is direct and how it is affected by alterations of Hsp90. Further analyses will also have to establish the stoichiometry of the complex and the proportion of Ste11 that is associated with Hsp90 at any given time. Interestingly, the effects of mutating *HSP90* in yeast are mirrored by pharmacological experiments with the Hsp90 "drug" geldanamycin (or herbimycin A, another ansamycin) in vertebrate cells. Raf-1 is degraded when cells are treated with this compound (Schulte *et al.*, 1995–1997; Schneider *et al.*, 1996; Stancato *et al.*, 1997). Similar effects have been reported for the glucocorticoid receptor, another Hsp90 substrate (Whitesell and Cook, 1996; Czar *et al.*, 1997; Segnitz and Gehring, 1997). Although accumulation of Raf was apparently not affected in *Drosophila* strains with *HSP83* mutations (van der Straten *et al.*, 1997), it should be pointed out that the severity of the effect also depends on the mutation in our system. Recently, Errede and her collaborators have obtained

Table 1. Summary of the phenotypes of the Hsp90 mutants

	G_1 arrest (α-factor)		G_1 arrest (Ste 11ΔN)		*FUS1-LacZ* expression		*FUS1-HIS3* expression	
Hsp82 mutants	30°C	21°C	30°C	21°C	30°C	21°C	30°C	21°C
Hsp82 wt	+++	+++	+++	+++	+++	+++	+++	+++
10% Hsp82 wt	−	−	NA	NA	+/−	+/−	ND	ND
Hsp82 G313N	−	++	+/−	+++	+/−	++	−	++
Hsp82 T525I	+++	+++	+++	+++	ND	ND	ND	ND
hHsp90β	−	−	+/−	+++	+/−	+/−	−	−

Note that this table also includes all the results presented in Figures 1, B and C, 2, and 3A, which were obtained by culturing cells at 30°C. ND, not done; NA, not applicable. +++, ++ and +/− indicate full, partial, and weak responses, respectively.

results that support our conclusions. They could notably demonstrate with a temperature-sensitive Hsp82 mutant (Nathan and Lindquist, 1995) that the accumulation of newly synthesized Ste11 depends on continuous Hsp90 function (Buehrer, Rhodes, Rutherford, and Errede, unpublished data).

The reduced accumulation of Ste11 (and possibly Ste7) might be sufficient to account for the mutant phenotype. Since it is technically difficult to measure the specific activity of Ste11, we cannot exclude that Ste11 also requires Hsp90 to reach its full enzymatic activity. The residual number of Ste11 molecules in Hsp90 mutant strains might well be sufficient, but they may have a lower specific activity. In the case of geldanamycin-treated vertebrate cells, specific activity of Raf appears to remain unchanged (Stancato *et al.*, 1997) whereas in *Drosophila* its specific activity appeared to be affected by *HSP83* mutations (van der Straten *et al.*, 1997).

The Hsp90 mutant strains that we have tested are not completely defective in Ste11 activity. Unlike *ste11* deletion strains, they are able to form shmoos in re-

Table 2. Yeast strains

Strain	Genotype[a]	Source
HH1-KAT6	*MAT*α *ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1* Δ*hsc82::LEU2* Δ*hsp82::LEU2* / *GAL1-hHSP90*β*-CEN/ ARS-HIS3* [pGAL1-hhsp90]	S. Lindquist (described in Palmer *et al.*, 1995)
HH1a[b]	*MATa ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1* Δ*hsc82::LEU2* Δ*hsp82::LEU2*	This article
HH1a-pHCA/Hsp82wt	HH1a / *HSP82-CEN/ARS-HIS3* [pHCA/Hsp82]	This article
DP120	HH1a / *HSP82-2*μ*-URA3* [p2U/Hsp82]	This article
DP121	DP120 / *fus1::HIS3*	This article
DP122	HH1a / *fus1::HIS3* / *HSP82-CEN/ARS-TRP1* [pTCA/ Hsp82]	This article
DP123	HH1a / *fus1::HIS3* / *HSP82 G313N-CEN/ARS-TRP1* [pTCA/Hsp82 G313N]	This article
DP124	HH1a / *fus1::HIS3* / *hHSP90*β*-2*μ*-TRP1* [p2TG/hHsp90β]	This article
HH1a-p2G/Hsp82wt	HH1a / *HSP82-2*μ [p2G/Hsp82]	This article
HH1a-p2G/Hsp82 G313N	HH1a / *HSP82 G313N-2*μ [p2G/Hsp82 G313N]	This article
HH1a-p2G/hHsp90β	HH1a / *hHSP90*β*-2*μ [p2G/hHsp90β]	This article
HH1a-p2TG/flag.Hsp82wt	HH1a / *flag.Hsp82-2*μ*-TRP1* [p2TG/flag.Hsp82wt]	This article
JC6a[b]	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* Δ*hsc82::LEU2* Δ*hsp82::LEU2* Δ*pep4::HIS3*	S. Lindquist
JC6a-Hsp82	JC6a / *HSP82-CEN/ARS-TRP1* [pTCA/Hsp82]	This article
JC6a-Hsp82 G313N	JC6a / *HSP82 G313N-CEN/ARS-TRP1* [pTCA/Hsp82 G313N]	This article
JC6a-hHsp90β	JC6a / *hHSP90*β*-2*μ*-TRP1* [p2TG/hHsp90β]	This article
RMY326	MATa *his3 leu2-3,112 trp1-1 ura3-52*	R. Movva
E929-6C-20	E929-6C-0 *ste11-*Δ*6*	Rhodes *et al.*, 1990

[a] Episomes are indicated after a slash with the name of the plasmid in brackets.

[b] HH1a and JC6a represent related genotypic backgrounds of many strains rather than real strains (they are not viable without Hsp90 function being provided, for example, by a plasmid).

sponse to pheromone, and they can mate albeit with reduced efficiency (our unpublished results). The hyperphosphorylation of Ste7 that occurs at a lower level even in Hsp90 mutant strains further corroborates that there is residual Ste11 activity. This is either due to a pathway that allows Ste11 maturation/stabilization to proceed partially in an Hsp90-independent manner or to residual activity of our panel of Hsp90 mutants. Indeed, Hsp90 mutants that are both viable and completely defective for this specific function may be difficult to find. Along with the fact that there are two genes for Hsp90 in *S. cerevisiae*, this residual Ste11 activity probably explains why *HSP90* was never found in screens for sterile mutants.

Is Ste11 the Only Substrate of Hsp90 in the Pheromone-signaling Pathway?

Both biochemical evidence and results obtained with the yeast two-hybrid system have led to the view that Ste11, Ste7, Fus3/Kss1, and other components of the pheromone pathway are all tethered together by Ste5. Ste5 may serve as a scaffold to maintain the different kinases and their substrates in a macromolecular signal transduction complex, thereby ensuring specificity and efficiency (for reviews, see Elion, 1995; Leberer *et al.*, 1997). This illustrates that the notion of a linear signal transduction from upstream to downstream components, as derived from genetic epistasis experiments, is too simplistic. Moreover, it does not take into account that additional factors such as molecular chaperones could be required for the maturation of the individual components and/or the multiprotein complex. Two linked hypotheses are worth considering in this context: 1) Hsp90 chaperones the dynamic assembly of this multiprotein signaling complex; 2) Hsp90 is required for the maturation/stabilization of additional signaling molecules. Note that Hsp90 does not have to be a stable component of these complexes; it might only transiently interact with Ste11 and/or other proteins.

To address the first hypothesis the tools have yet to be developed. Using the yeast two-hybrid assay that relies on interactions of chimeras in the nucleus, we have not seen any differences in Hsp90 mutant strains for the interactions of Ste5 with Ste11 or Ste7 (our unpublished results). However, it will ultimately be necessary to characterize the complex formed of the endogenous wild-type proteins, a technically daunting task.

Regarding the second hypothesis, the reduced Ste7 levels are compatible, but not more, with an interaction of Ste7 with Hsp90. The differential behavior of certain Hsp90 mutants, notably hHsp90β, at different temperatures in different assays (see Table 1) might also support such an assumption. While hHsp90β allows signaling by the constitutive Ste11ΔN at low temperature, it fails to allow pheromone to signal through the complete pathway. Interestingly, Ste5 overexpression suppresses the signaling defect of Hsp90 mutant strains, but only biochemical experiments will be able to elucidate how this increases the efficiency of the signaling complex. Taking these observations as guidelines, the interaction of Hsp90 with signaling molecules both upstream and downstream of Ste11 as well as Ste5 will have to be examined directly.

Hsp90 Requirement in Other MAPK Pathways

Other MAPK-signaling pathways in yeast may also depend on Hsp90. While the cell wall integrity pathway does not appear to be affected in our Hsp90 mutant strains (our unpublished results), other pathways await examination. This will be particularly interesting for the three other pathways that are known to share Ste11: one of the two osmoregulatory pathways (Posas and Saito, 1997), the invasive growth response of haploid cells, and pseudohyphal development of diploids (see Herskowitz, 1995; Levin and Errede, 1995; Schultz *et al.*, 1995). In this context it is noteworthy that the growth arrest/"toxicity" induced by Ste11ΔN appears to be due to its functions in both the pheromone and the high osmolarity response pathways (Posas and Saito, 1997). Since Hsp90 mutant strains are at least partially refractory to the Ste11ΔN toxicity, we speculate that Hsp90 may be required for Ste11 function in both pathways.

Genetic Dissection of Different Hsp90 Functions

Previous studies had demonstrated that it is possible to selectively abolish specific dispensable functions of Hsp90 without compromising its ability to ensure viability in yeast; specifically, a variety of *HSP82* mutations result in a defect in the regulation of steroid receptors or v-Src or folding of p53 in yeast (Picard *et al.*, 1990; Bohen and Yamamoto, 1993; Xu and Lindquist, 1993; Bohen, 1995; Nathan and Lindquist, 1995; Blagosklonny *et al.*, 1996; Fang *et al.*, 1996; Nathan *et al.*, 1997). We have now considerably extended this theme by showing that even subtle point mutations can discriminate between the Hsp90 requirements in two different signaling pathways. Some mutants, like the Hsp82 point mutant G313N, are defective in both steroid receptor and pheromone signaling. Another point mutant, T525I, is only defective in steroid receptor signaling while the converse is true for human Hsp90 (this article and our unpublished results). Moreover, G313N has a different temperature sensitivity for several Hsp90 functions: at room temperature, only hormone binding of steroid receptors is defective (Bohen, 1995) whereas viability (Bohen and Yamamoto, 1993) and pheromone signaling are only lost upon increasing the temperature to 37°C and

30°C, respectively. A deletion analysis of *HSP82* has proven of limited use in assigning specific functions to individual domains of Hsp90 (Louvion *et al.*, 1996). Only two regions, the eukaryote-specific N-terminal charged domain and the C-terminal conserved pentapetide, could be deleted without affecting viability. These two portions of Hsp82 are also dispensable for Hsp90 function in pheromone signaling (Louvion *et al.*, 1996). By ensuring viability with human Hsp90 (hHsp90β), which cannot promote pheromone signaling, it might nevertheless be possible to map the domains of Hsp82 that are specifically required for its function in pheromone signaling. In such a system, even coexpressed Hsp82 mutants, which fail to provide the viability function, might be able to restore pheromone signaling. Additional insights could be gained by examining a series of chimeras between yeast Hsp82 and human Hsp90β.

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