MOLECULAR AND CELLULAR BIOLOGY, July 1995, p. 3917–3925 Vol. 15, No. 7
0270-7306/95/$04.00+0

Mutational Analysis of Hsp90 Function: Interactions with a Steroid Receptor and a Protein Kinase

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Received 16 February 1995/Returned for modification 22 March 1995/Accepted 21 April 1995

Hsp90 is a protein chaperone whose functions are focused on a specific set of target proteins. The nature of Hsp90's interactions with these proteins is poorly understood. To provide tools for examining these interactions, we have isolated eight broadly distributed temperature-sensitive (ts) point mutations in the Hsp90 gene (*HSP82*) of *Saccharomyces cerevisiae*. The mutants fall into two distinct classes. One has a classic ts phenotype, with nearly wild-type activity at 25°C and a precipitous loss of function at 34°C. The remaining seven mutants, in contrast, cause a general reduction in Hsp90 function and are ts because they do not provide the high level of function required for growth at high temperatures. The effects of these mutants on two target proteins, a transcription factor (glucocorticoid receptor) and a tyrosine kinase ($pp60^{v\text{-}src}$), provided several insights on Hsp90 function. First, Hsp90 is not only required to help the glucocorticoid receptor achieve a hormone-activable state, it is continuously required to maintain that state. Second, Hsp90's function in the maturation of $pp60^{v\text{-}src}$ involves separable roles in protein accumulation and kinase activation. Thus, Hsp90 is an integral component of both the steroid receptor and kinase signaling pathways. Finally, all eight point mutants affect the activation of both the glucocorticoid receptor and $pp60^{v\text{-}src}$, indicating that Hsp90 promotes the activity of these very different target proteins through common mechanisms.

When cells are exposed to elevated temperatures or other environmental stresses, heat shock proteins are rapidly and dramatically induced. Many heat shock proteins function as chaperones and play important roles in normal growth as well as in stress tolerance. At normal temperatures, they promote the folding, oligomeric assembly, and translocation of polypeptides by stabilizing folding intermediates and preventing off-pathway interactions. At high temperatures, their expression is increased to prevent the aggregation of heat-denatured proteins and to facilitate proper refolding (33).

One heat shock protein, Hsp90, has only recently been recognized as a molecular chaperone. In vitro, Hsp90 functions as a general chaperone, suppressing the aggregation of unfolded citrate synthase (55) and purified, aggregation-prone casein kinase II (28). Moreover, in the presence of Hsp70 and ATP, Hsp90 promotes the reactivation of heat-denatured firefly luciferase (45).

In vivo, at normal temperatures, the chaperone activities of Hsp90 seem focused on a diverse but restricted group of target proteins. Thus, Hsp90 forms strong associations with steroid hormone receptors (10, 36, 42, 44), basic helix-loop-helix transcription factors (46), oncogenic tyrosine kinases (8), and normal cellular serine-threonine kinases (28, 37, 52, 54) and yet shows little or no tendency to associate with closely related proteins. That is, Hsp90 associates with glucocorticoid receptors (GR), progesterone receptors (PR), and estrogen receptors but not with retinoic acid receptors and thyroid hormone receptors (10, 14, 15, 36, 42, 44). Similarly, one nonreceptor tyrosine kinase, $pp60^{v\text{-}src}$, is highly dependent on Hsp90, while another, $pp160^{v\text{-}abl}$, is not (56).

Even those target proteins that are known to require a high level of Hsp90 function vary widely in the nature of their association with this chaperone. For example, the association of $pp60^{v\text{-}src}$ with Hsp90 is restricted to a brief period immediately after $pp60^{v\text{-}src}$ is synthesized (8), while the interaction with GR is long-lived and has acquired regulatory significance (4, 34, 35, 51). Furthermore, when isolated from intact cells, Hsp90 target proteins are found to be associated with several proteins in addition to Hsp90 (4, 8, 17–19, 21, 35, 39, 49–52, 54). While some of these proteins are common to most target proteins, others are unique. Thus, p59 is part of Hsp90-steroid receptor complexes (35, 51), while p50 is a component of Hsp90-Raf (52) and Hsp90-$pp60^{v\text{-}src}$ (8) kinase complexes.

The roles of most members of these heteromeric complexes are unclear. However, recent in vitro experiments suggest that some of these proteins play a role in establishing Hsp90-target protein interactions (19, 21, 50). In fact, the presence of one of these proteins, Hsp70, is required for the reconstitution of Hsp90-GR and Hsp90-PR complexes in rabbit reticulocyte lysates (19, 50). Moreover, these reconstitution experiments demonstrate that Hsp90 binds to GR and PR only in association with other members of the heteromeric complex (21, 39, 50). A more detailed examination of Hsp90-PR complexes indicates that they are dynamic (49) and that PR cycles through associations with several different preformed Hsp90 complexes (21, 49). Although Hsp90-kinase complexes have not been studied as thoroughly as steroid receptor complexes, they may assemble in a similar manner. In vitro reconstitution of all of these complexes requires components of a rabbit reticulocyte lysate, Mg^{+2}-ATP, and K^+, and all of the complexes are stabilized by molybdate (17, 18, 50, 52).

We have taken advantage of yeast genetic methods to derive tools for the analysis of Hsp90-target protein interactions. The approach is validated by the remarkable conservation of Hsp90 functions throughout the eukaryotic lineage. Hsp90 is essential for viability in *Saccharomyces cerevisiae* (5), and yet the human Hsp90 protein supports near wild-type growth rates in yeast cells lacking endogenous Hsp90 (27, 34). Moreover, yeast Hsp90 forms functional interactions with several vertebrate

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target proteins, including GR (34) and pp60$^{v\text{-}src}$ (56). Finally, components of the larger macromolecular complex in which Hsp90 functions are conserved between *S. cerevisiae* and vertebrates (11).

Here we report the isolation of a series of amino acid substitutions in *S. cerevisiae HSP90* that confer a temperature-sensitive (ts) growth phenotype on cells lacking the wild-type protein. Given that higher levels of Hsp90 function are required for growth at high temperatures, theory predicts that two different classes of mutants might be obtained. Indeed, of the eight single amino acid substitutions isolated, only one behaves as a classic ts mutant, with wild-type function at low temperatures and virtually no activity at high temperatures. The remaining mutations cause general reductions in Hsp90 function, and cells expressing them are ts because the mutant proteins do not provide enough activity for high-temperature growth.

We have used these mutants to examine the interaction between Hsp90 and two very different target proteins. The seven mutants with reduced function at all temperatures have amino acid substitutions broadly distributed through the protein. These mutants were employed to determine how mutations in different regions of the protein affect the ability of Hsp90 to functionally interact with GR and pp60$^{v\text{-}src}$.

The classic ts mutant precipitously loses activity during a shift to high temperature. This mutant was employed to examine the function of Hsp90 in the mature aporeceptor complex. Previous in vivo studies established that Hsp90 is required for GR to achieve a hormone-activable state (34), but once this state has been achieved, the role of Hsp90 was uncertain. In vitro, when the GR-Hsp90 aporeceptor complex is disrupted, GR's ability to bind DNA is increased (31, 41) but its ability to bind hormone is decreased (7). Reconstitution of the GR-Hsp90 complex in reticulocyte lysate restores GR's hormone binding activity and represses its DNA binding activity (43). Whether the continued presence of Hsp90 in the aporeceptor complex is important in vivo and, if so, whether Hsp90 acts to repress or promote GR-mediated transcription are not known. Temperature shift experiments with this mutant provided a means for answering these questions.

MATERIALS AND METHODS

Plasmid and strain construction. All strains were derivatives of W303 (*ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1*; R. Rothstein). To avoid gene conversion between plasmid-borne mutants and chromosomal *HSP82* and *HSC82* genes, new mutations were created to eliminate most of the chromosomal coding sequences. The 5-kb *Bam*HI fragment of *HSC82* from pUTX203 (5) was subcloned into the Bluescript vector (Stratagene) and the *Bcl*I-*Sty*I fragment was replaced with the *LEU2* gene from pUTX123 (5) to create the *HSC82* disruption plasmid pBK1. The *Hin*dIII-*Cla*I fragment of *HSP82* from pUTX17 (5) was moved into pVZ1 (Stratagene) and the *Sty*I fragment was replaced with the *LEU2* gene to create the *HSP82* disruption plasmid pBK2.

The haploids ΔCLD82α and ΔPLD82**a** were created by transforming linear DNA from pBK1 and pBK2 into W303α and W303**a**, respectively. Gene replacements were obtained by selecting for leucine prototrophy. ΔPLD82**a** was then transformed with a wild-type *HSC82* expression plasmid, pKAT6, created by inserting a *Bgl*II linker into the *Cla*I site of pUTX203 and subcloning the *Bgl*II-*Sph*I coding fragment into YEP24 (6). The resulting strain, ΔPLD82/ECU**a**, was mated to ΔCLD82α, the heterozygous diploid ΔPLD82**a**/ΔCLD82α was sporulated, and Leu$^+$ Ura$^+$ spores from tetrads with a 2:2 segregation of LEU2 were selected. These haploid *hsc82 hsp82* double deletion strains, ΔPCLD82**a** and ΔPCLD82α, which carried the wild-type *HSC82* plasmid were then mated to form the homozygous diploid ΔPCLD**a**/α. To reduce the chances of recovering extraneous genomic mutations, a mutant screen was used for this diploid.

HIS3- and *TRP1*-marked expression vectors (pHGPD/P82 and pTGPD/P82, respectively) were constructed by inserting the 0.7-kb *Eco*RI-*Bam*HI fragment containing the glyceraldehyde-3-phosphate dehydrogenase promoter from p2Hg (34) into pRS313 and pRS314 (48), respectively. The 3-kb *Bam*HI fragment containing the *HSP82* coding region from pTT8 (34) was then inserted behind the glyceraldehyde-3-phosphate dehydrogenase promoter.

The galactose-inducible GR plasmid, p2HGAL/GR/CYC, was constructed by inserting the *Bam*HI fragment from p2Hg/N795 (34) containing GR coding sequences into a pRS423-based vector (12), p2HGAL/CYC. This vector contains the *GAL1-10* promoter (22) and 236 bases of 3′ untranslated sequences from the *CYC1* gene (pGYC1; gift of R. Russnak). The GR^{525} expression plasmid, p2HGAL/CGR/CYC, was constructed by inserting the *Bam*HI fragment from p2HG/N525 (34) into p2HGAL/CYC. To construct p2A/GRGZ, the 8-kb *Kpn*I fragment from pHCA/GRGZ (gift of K. Yamamoto), which contains the GR coding sequence under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter as well as a *lacZ* reporter gene under the control of three glucocorticoid response elements, was inserted into pL909 (gift of R. Keil). The reporter plasmid, pSX26.1, has been described elsewhere (34). The v-*src* expression plasmid, Y316v-src, was obtained from D. Morgan (29).

Yeast and bacterial transformants were obtained by lithium acetate (20) and calcium chloride (25) methods, respectively, or by electroporation with a Gene Pulser (BioRad).

Yeast media. Cells were cultured in SD, SR, or SGal (20 g of glucose, raffinose, or galactose, respectively, per liter with 5 g of ammonium sulfate per liter and 1.7 g of yeast nitrogen base without amino acids per liter, supplemented with essential amino acids and nucleotides); YPD (10 g of yeast extract per liter, 20 g of Bacto Peptone per liter, 20 g of glucose per liter, and 2 mg of adenine sulfate per liter); YPGlycerol (substitution of 3% glycerol for the glucose in YPD); or 5-FOA (1.7 g of yeast nitrogen base without amino acids and ammonium sulfate per liter, 1 g of proline per liter, 20 g of glucose per liter, 10 mg of uracil per liter, and 600 mg of 5-fluoro-orotic acid per liter, supplemented with essential amino acids and nucleotides [26]). Solid media contained 20 g of Bacto agar per liter.

Hydroxylamine mutagenesis. Hydroxylamine mutagenesis was performed essentially as described previously (38, 47), except that hydroxylamine solutions were adjusted to pH 6.25 or 7.0 to achieve different mutagenesis efficiencies. Ten-microgram aliquots of pHGPD/P82 DNA were incubated in 500 μl of 1 M hydroxylamine for 75 min at 75°C. DNA was extracted with phenol-chloroform (1:1) and recovered by ethanol precipitation. Mutagenesis efficiencies were assayed in *Escherichia coli* KC8 by the number of *his3* null mutations generated (47). Two pools of DNA mutagenized at pH 7.0 produced 1.8 and 2.7% *his3* mutations, and one pool mutagenized at pH 6.25 produced 5.4%. *hsp82* mutants were isolated from each pool.

Screen for *hsp82* ts mutants. Mutagenized pHGPD/P82 DNA (1 to 5 μg) was transformed into ΔPCLD**a**/α. Transformants were plated onto SD minus histidine and then either patched or replica plated onto SD minus histidine at 25°C, 5-FOA at 25°C, 5-FOA at 37°C, and YPGlycerol at 25°C. The 5-FOA plating selected for cells that had lost the wild-type *HSC82* plasmid (2). The YPGlycerol plating eliminated extraneous respiratory mutations, which exhibit a ts phenotype on 5-FOA. Transformants that did not grow on 5-FOA at 37°C but that grew under all other conditions were selected. Colonies (from the plate with SD minus histidine at 25°C) were streaked onto 5-FOA and rescreened for temperature sensitivity on SD minus histidine.

To ensure that the ts phenotypes were due to the mutagenized DNA, plasmids were recovered (16), transformed into ΔPCLD**a**/α, and rescreened. The *HSP82* coding sequences were then subcloned from the mutagenized plasmids into pTGPD, transformed into ΔPCLD**a**/α, and rescreened. The mutations also conferred a ts phenotype on haploid strains, and *MAT***a** strains were used for all subsequent experiments.

HSP82 coding regions were sequenced with the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and a model 370A automated sequencer (Applied Biosystems) or with the Sequenase 2.0 kit (United States Biochemicals) and ^{35}S-dATP (Amersham).

Temperature ramp. A temperature ramp was constructed (1, 24) with aluminum plates, and it was connected at each end to aluminum blocks in contact with a model CN-2000 programmable temperature controller (Omega). The temperature controllers were set to 51°C and 25.5°C. Further cooling was achieved by circulating 40% polyethylene glycol cooled to 10°C in a model F-3K refrigerated bath and circulator (Haake) through the block at the cool end. Yeast cultures (2×10^6 cells per ml in YPD) were spotted across a YPD (containing 12 mg of tetracycline per liter) agar slab (with a 48-pin replicator; V&P Scientific Inc.) in an aluminum pan and incubated on the ramp for 40 h. Temperatures were monitored across the gradient by inserting the probes of a model OM205 data logger (Omega) between the bottom aluminum sheet and the agar-containing pan. This method produced a highly reproducible temperature gradient, but it should be noted that the temperatures recorded by the probes do not necessarily correspond to the actual temperatures at the agar surface on which the cells are growing.

Induction of pp60$^{v\text{-}src}$ expression. Cells were grown to a concentration of ~2×10^6 cells per ml in SR minus uracil, collected by centrifugation, and transferred to SGal minus uracil for 6 h. For the analysis of the G170D mutant, 34°C was used as the nonpermissive temperature (instead of 37°C) to minimize the loss of viability. Viability was assessed by spotting serial dilutions of the cultures onto SD-minus-uracil plates at 25°C.

Induction and activation of GR. Constitutive expressers were grown to a concentration of ~2×10^6 cells per ml in SD minus adenine. Deoxycorticosterone (DOC) (10 μM) was added for 1 h to activate the receptors. For galactose induction, cells were grown to a concentration of ~2×10^6 cells per ml in SR minus histidine and uracil. The cells were collected by centrifugation and transferred to SGal minus histidine and uracil for 16 h to induce GR. Cells were then

transferred to SD minus histidine and uracil containing 10 μM DOC for 1 h to stop GR synthesis and activate the receptors. For experiments comparing different mutants, 37°C was employed as a general nonpermissive temperature. For detailed analysis of the G170D mutant, incubations were performed at 34°C to minimize the possible loss of viability. Similar results were obtained with this mutant at both temperatures.

Measurement of β-galactosidase activity. Care was taken to ensure that all cells were at the same stage in the growth curve at the time of the assay (2×10^6 cells per ml). Cells (5×10^7 cells per sample) were collected by centrifugation, washed once with H_2O, and frozen at −80°C. Thawed cells were resuspended in 150 μl of lysis buffer [0.1 M potassium phosphate (pH 7.8), 20% glycerol, 1 mM dithiothreitol, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μg of leupeptin per ml, 2 μg of aprotinin per ml, 2 μg of pepstatin A per ml] and lysed with glass beads (425 to 600 μm) by agitation on a multitube vortexer (VWR Scientific) for 3 min at 4°C. β-Galactosidase activity was measured with the Galactolight Kit (Tropix) and normalized to the protein concentration of the lysate, which was determined by the BioRad assay.

Western blot (immunoblot) analysis. Cells (10^8) were collected by centrifugation, washed with H_2O, and resuspended in 200 μl of ethanol containing 2 mM phenylmethylsulfonyl fluoride. Cells were lysed by agitation with glass beads for 5 min at 4°C. Proteins were precipitated at −35°C, dried in a vacuum in a SpeedVac Concentrator (Savant), and resuspended in sample buffer (65 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, and bromophenol blue).

Proteins separated on SDS–7.5% polyacrylamide gels were transferred to Immobilon-P membranes (Millipore) and stained with Coomassie blue. Blots were blocked with 5% nonfat dehydrated milk in phosphate-buffered saline (180 g of NaCl per liter, 4 g of KH_2PO_4 per liter, 23 g of Na_2HPO_4 per liter, 4 g of KCl per liter, 0.02% thimerosal) and incubated with primary antibody for 1 h, with affinity-purified rabbit anti-mouse immunoglobulin G antibody (Cappel) for 1 h, and finally with protein A-conjugated horseradish peroxidase (Boehringer Mannheim) for 1 h. Immune complexes were visualized with the ECL reagent (Amersham). Antibody 4G10 (Upstate Biotechnology Incorporated) was used to detect phosphotyrosine residues, antibody LA074 (Quality Biotech) was used to detect $pp60^{v\text{-}src}$, and antibody BuGR2 (Affinity Bioreagents) was used to detect GR.

RESULTS

Isolation of *hsp82* ts mutants. In the yeast *S. cerevisiae*, Hsp90 is coded for by two nearly identical, functionally indistinguishable genes, *HSC82* and *HSP82* (5). We used the plasmid shuffle technique (47) to isolate point mutations in the *HSP82* gene that confer a ts growth phenotype on cells lacking both wild-type genes (Fig. 1A; see also Materials and Methods). Briefly, a mutagenized *hsp82* expression plasmid was transformed into a yeast strain whose only source of Hsp90 was provided by another plasmid. Transformants that had acquired the mutagenized plasmid were allowed to lose the wild-type plasmid and were then screened for ts growth. To confirm that the ts phenotypes were due to Hsp90 mutations, the coding regions were subcloned into new vectors under the control of a constitutive promoter and rescreened. Twenty confirmed mutants were sequenced to identify the point mutations responsible for the ts phenotype.

All of the mutants contained G→A or C→T transitions, as expected for hydroxylamine mutagenesis. Eight contained multiple mutations; twelve contained single substitutions, eight of which were unique. The unique single amino acid substitutions were broadly distributed through the Hsp90 coding region (Fig. 1B). Threonine at amino acid residue 22 was replaced by isoleucine (T22I), alanine at residue 41 was replaced by valine (A41V), glycine at residue 81 was replaced by serine (G81S), threonine at residue 101 was replaced by isoleucine (T101I), glycine at residue 170 was replaced by aspartate (G170D), glycine at residue 313 was replaced by serine (G313S), glutamate at residue 381 was replaced by lysine (E381K), and alanine at residue 587 was replaced by threonine (A587T). All were recessive to the wild type. Further analysis was restricted to these single amino acid substitutions, with the constitutive promoter employed to govern their expression.

Growth properties of the *hsp82* mutants. Two of the mutants, G170D and A587T, had wild-type doubling times (1.6 h)

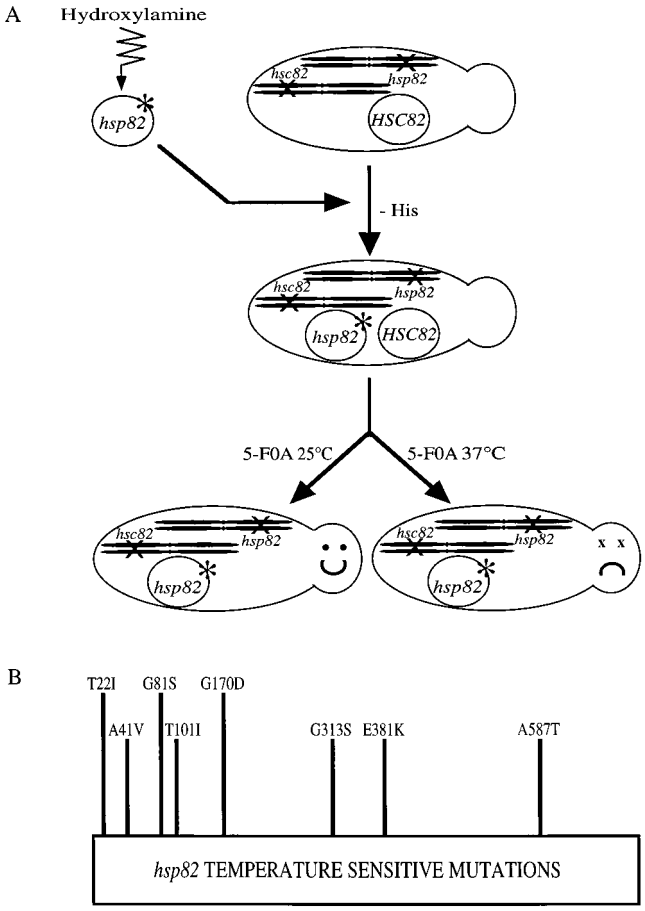

FIG. 1. Isolation of *hsp82* mutants. (A) Strategy for the isolation of ts mutations in the *S. cerevisiae HSP82* gene. An *HSP82* expression plasmid, pHGPD/P82, was mutagenized with hydroxylamine and transformed into ΔPCLD**a**/α, a diploid yeast strain that is homozygous for deletion mutations in both Hsp90 genes (*hsp82* and *hsc82*) and is rescued from lethality by a *URA3*-marked plasmid expressing wild-type Hsp90 (*HSC82* plasmid pKAT6). Transformants were patched onto 5-FOA-containing medium at 25 and 37°C to select for colonies that had lost the wild-type *HSC82* plasmid and that were ts for growth. (B) The eight single amino acid substitutions in the *HSP82* gene which conferred a ts phenotype are indicated. Eight additional mutants contained multiple amino acid substitutions, six of which involved glycine 170. They were G170E-D180N, G170D-P275S, G170D-V429I, G170D-D527Y, G170D-L62F-P275S, G170D-K514N-E515K-E517K-G518S, D370N-G655D, and T257M-S478F.

in liquid medium at 25°C. All of the remaining mutants exhibited some growth defects even at 25°C, with the most severe, T22I and E381K, growing at only half the rate of wild-type cells. When liquid cultures of log-phase cells were shifted to 37°C, five of the mutants doubled two to three times before arresting. The remaining three mutants, G170D, G313S, and E381K, stopped dividing before a single division was completed (Fig. 2A). The G313S mutant, however, is distinguished from G170D and E381K (and all of the other mutants) by having an extremely elongated morphology at 25°C (data not shown).

To examine the ts phenotype of the *hsp82* mutants in more detail, a temperature ramp was constructed. This apparatus establishes a continuous temperature gradient and was used to assess growth on solid medium over a broad range of temperatures (28.5°C to 39.5°C). Cells expressing wild-type Hsp90 grew to 38.5°C. None of the mutants grew at above 36°C (Fig. 2B). The mutants which stopped dividing most rapidly on a

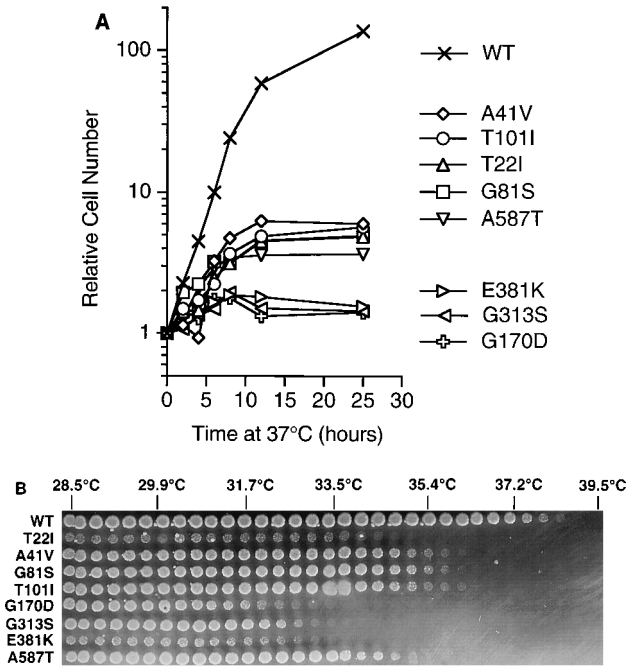

FIG. 2. The ts growth phenotype of the *hsp82* mutants. (A) Growth curves. Mid-log-phase cultures of wild-type and *hsp82* mutant strains grown at 25°C were diluted to a density of 10^5 cells per ml in YPD medium which had been equilibrated to 37°C. At various times, aliquots were removed from the 37°C cultures and counted with a hemocytometer. The data shown are the averages of two independent experiments. WT, wild type. (B) Maximal growth temperature. Mid-log-phase cultures of wild-type and *hsp82* mutant strains grown at 25°C were spotted onto an agar slab containing YPD medium. A temperature gradient was applied to the slab, and the cells were allowed to grow for 40 h. Maximum growth temperatures varied by less than 0.2°C in two independent experiments. WT, wild type.

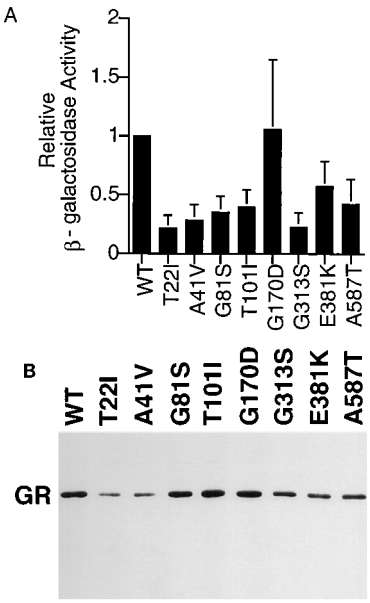

FIG. 3. GR activity and accumulation in the *hsp82* mutants at 25°C. (A) Assessment of GR activity. Wild-type and *hsp82* mutant strains were transformed with a constitutive GR expression plasmid which also contains *lacZ* sequences under the control of glucocorticoid response elements. Mid-log-phase cells were treated with 10 μM DOC for 1 h. β-Galactosidase activities were normalized to the total protein concentration in the cell lysate and are expressed as percentages of the activity observed in wild-type (WT) cells. The data shown are the means and standard deviations (error bars) of at least three independent experiments. (B) Accumulation of GR protein. Total cellular proteins were separated on an SDS–7.5% polyacrylamide gel and transferred to an Immobilon-P membrane. Equal sample loading was confirmed by Coomassie blue staining (not shown), and the blot was reacted with an antibody specific for GR. WT, wild type.

shift to 37°C, G170D, G313S, and E381K, exhibited the lowest maximum growth temperatures (<33°C). The two mutants with the slowest doubling times at 25°C, T22I and E381K, grew poorly over the entire temperature range. Three mutations clustered in a 60-amino-acid stretch of the protein (codons 41, 81, and 101) caused cells to stop growing at the same point, 36°C. For the remaining mutants, the maximum growth temperature bore no clear relationship to the location of the substitution.

The ts phenotypes are not due to reduced Hsp90 accumulation. Low levels of Hsp90 are sufficient for growth at 25°C, but high levels are required at 37°C (5). Therefore, in theory, two types of mutations might produce a ts phenotype: (i) mutations that cause a general reduction in Hsp90 function, lowering protein concentration or activity to the point at which it can only sustain life at low temperatures (5), and (ii) mutations that cause the protein to unfold at high temperatures but that provide normal, or nearly normal, activity at low temperatures (classic ts mutations).

To determine if any of the mutations lower the concentration of Hsp90, we examined proteins in cells growing at 25°C and cells shifted to 37°C for 1 to 24 h. A 10- to 20-fold reduction in Hsp90 concentration is required to lower the maximum growth temperature of yeast cells to 37.5°C (5, 53). The G170D and T22I mutations reduced Hsp90 accumulation slightly at both temperatures, but Hsp90 levels remained far above those required to sustain viability at 37°C (data not shown). Because the maximum growth temperatures of both mutants are well below 37°C, their ts phenotypes cannot simply be due to reduced levels of protein. None of the other mutants reduced Hsp90 accumulation at either temperature.

Since interactions that stabilize the folded state of a protein are highly cooperative, proteins unfold and lose activity over a narrow temperature range (32). Classic ts mutations reduce the temperature at which this transition occurs. Because six of our mutations reduced growth over a broad range of temperatures, it seems unlikely that they are classic ts mutations. To confirm their classification and to investigate the character of the two mutants that exhibited wild-type growth at 25°C, a more sensitive assay was required.

Seven of the eight mutations reduce GR activity at normal temperatures. The mammalian GR, a well-characterized Hsp90 target protein, provides an extremely sensitive assay for Hsp90 function in yeast cells. Reductions in Hsp90 levels that have no effect on growth at 25°C can produce strong effects on GR activity (34). To assess the mutant proteins' ability to promote GR activity, each strain was transformed with a plasmid that constitutively expresses GR and carries a glucocorticoid-regulated *lacZ* reporter gene.

As expected, all six of the mutants with reduced growth rates at 25°C exhibited reduced GR activity at 25°C (Fig. 3A). This result confirms that the T22I, A41V, G81S, T101I, G313S, and E381K mutations compromise Hsp90 function at 25°C. Two of these mutants, T22I and A41V, accumulated less GR protein than did wild-type cells (Fig. 3B). Their reduced GR activity may be due, in whole or in part, to reduced GR accumulation. In all of the other mutants, the receptor accumulated at wild-type levels.

The GR assay also revealed that one of the two mutants with a wild-type growth rate at 25°C, the A587T mutant, had sub-

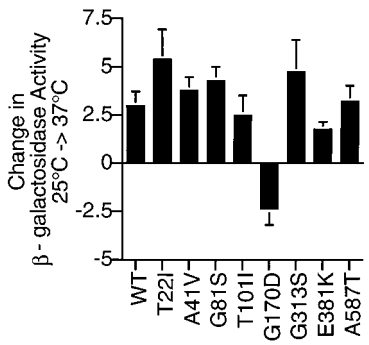

FIG. 4. GR activity in the *hsp82* mutants at 37°C. Wild-type (WT) and *hsp82* mutant strains analyzed in the experiment illustrated in Fig. 3 were grown to mid-log phase and resuspended in medium containing 10 μM DOC which had been preequilibrated at either 25 or 37°C. After a 1-h incubation, lysates were prepared and β-galactosidase activities were assessed. The data are presented as the fold differences between the activities at 25 and 37°C. An increase in activity at 37°C is indicated by a positive number, and a decrease in the activity at 37°C is indicated by a negative number. The data shown are the means and standard deviations (error bars) of at least three independent experiments.

stantially reduced Hsp90 function at 25°C. Thus, A587T functions are compromised over a very broad temperature range, from at least 25 to 35°C, its maximum growth temperature. Like the previous six mutations, A587T causes a general reduction in Hsp90 function. The other mutant with a wild-type growth rate at 25°C, G170D, displayed the same level of GR activity at 25°C as did wild-type cells (Fig. 3A).

G170D behaves like a classic ts mutant. Although the G170D mutant has wild-type GR activity at 25°C, it has one of the lowest maximum growth temperatures (Fig. 2B). The G170D protein, therefore, appears to lose activity over a narrow temperature range and may represent a classic ts mutant. This hypothesis was strengthened by the unique effects of increased temperature on the ability of G170D to promote GR activity.

In cells expressing wild-type Hsp90, GR activity increased approximately threefold when the temperature was increased to 37°C for 1 h (Fig. 4). The reason for this increase is unclear, but it is a general property of the receptor. (GR activity also increases in mammalian cells when the temperature is increased from 37 to 43°C [40].) The seven mutants with impaired Hsp90 function at 25°C behaved like wild-type cells in this respect. Although GR activity was reduced in these mutants relative to that of wild-type cells at 25°C, in all of them, GR activity increased two- to fivefold at 37°C. In striking contrast, GR activity was reduced in the G170D mutant (Fig. 4). This loss of activity was not an indirect effect of cell death. G170D cells and wild-type cells exhibited the same viability (colony-forming capacity) after 4 h at 37°C. Thus, G170D uniquely behaves as a classic ts mutant, with near wild-type function at low temperatures and loss of function at elevated temperatures. Moreover, this loss of activity does not rapidly translate into a loss of viability at nonpermissive temperatures.

Requirements for Hsp90 after GR synthesis and maturation. Once GR has been synthesized and matured to a hormone-activable state, it continues to associate with Hsp90 until the hormone triggers dissociation (4, 51). The G170D mutant provides a tool to investigate the significance of this extended interaction. Because GR is short-lived relative to the length of the assay (data not shown) and because we employed a constitutive promoter for GR expression, the experiment reported on in Fig. 4 does not distinguish the known requirement for Hsp90 during GR synthesis and maturation from a possible requirement in maintaining GR function. To focus on GR molecules that have already interacted with Hsp90 and achieved a hormone-activable state, GR coding sequences were placed under the control of the *GAL1* promoter. When cells growing in galactose medium are transferred to glucose medium, this promoter is strongly and immediately repressed (22).

Cells in the mid-log phase of growth were allowed to produce GR aporeceptor complexes in galactose at 25°C. Half of the cells were then transferred to glucose at 34°C to turn off GR synthesis and to inactivate the G170D protein. The other half were transferred to glucose at 25°C. As can be seen from Fig. 5A, the ability of the hormone to induce β-galactosidase activity was drastically reduced by the shift to 34°C in the G170D mutant.

This loss of activity in the G170D mutant was not an indirect effect on reporter gene expression. A C-terminal truncation mutant of GR (GR^{525}) which does not interact with Hsp90 and is constitutively active as a transcription factor (3) induced high levels of β-galactosidase activity in the G170D mutant at both 25 and 34°C (Fig. 5B). Moreover, when the *lacZ* gene was controlled by a heat shock response element, wild-type and G170D cells showed similar increases in β-galactosidase activity with a 39°C heat shock (data not shown). Finally, the loss of β-galactosidase induction could not be ascribed to a loss of GR protein. GR was actually more stable in G170D cells than in wild-type cells (Fig. 5C). Thus, after GR has been synthesized, functional Hsp90 is continuously required to maintain the receptor in a hormone-activable, transcriptionally competent state.

Mutations that reduce GR activity also reduce $pp60^{v\text{-}src}$ activity. Next, we asked whether mutations that affect Hsp90 function with one type of target protein, GR, would affect its function with a very different type of target protein, the tyrosine kinase $pp60^{v\text{-}src}$. Since $pp60^{v\text{-}src}$ expression is toxic in yeast cells (56), each of the mutants was transformed with a galactose-regulated v-*src* expression plasmid. Cells were grown in glucose and then transferred to galactose for 6 h to induce $pp60^{v\text{-}src}$ expression.

To assess the effects of the mutations on $pp60^{v\text{-}src}$ activity, total cellular proteins were reacted with an antibody specific for phosphotyrosine. Yeast cells have very low levels of endogenous tyrosine kinase activity, and at the levels of exposure we employed, no phosphotyrosine could be detected in cells that did not carry the $pp60^{v\text{-}src}$ expression plasmid (data not shown). As can be seen from Fig. 6A, all seven of the mutations that reduced GR activity at 25°C also reduced $pp60^{v\text{-}src}$ activity. The fact that all of the mutants affected both target proteins strongly suggests that the basic mechanisms by which Hsp90 acts to promote the activity of its target proteins are conserved for very different targets.

Separation of Hsp90's effects on $pp60^{v\text{-}src}$ activity and accumulation. Cells that exhibit reduced $pp60^{v\text{-}src}$ activity as a result of reduced wild-type Hsp90 expression also exhibit reduced $pp60^{v\text{-}src}$ accumulation (56). It has been difficult to determine, therefore, whether the loss of $pp60^{v\text{-}src}$ activity indicates a role for Hsp90 in $pp60^{v\text{-}src}$ kinase function per se or is simply a consequence of Hsp90's role in $pp60^{v\text{-}src}$ accumulation. Indeed, all seven of the Hsp90 point mutations which decreased $pp60^{v\text{-}src}$ activity at 25°C also decreased $pp60^{v\text{-}src}$ accumulation (Fig. 6A and B), and the degree to which kinase activity was affected generally correlated with the degree to which accumulation was affected. However, the G313S and A587T mutants did show distinct effects on kinase activity and accumulation. The G313S mutant accumulated more $pp60^{v\text{-}src}$

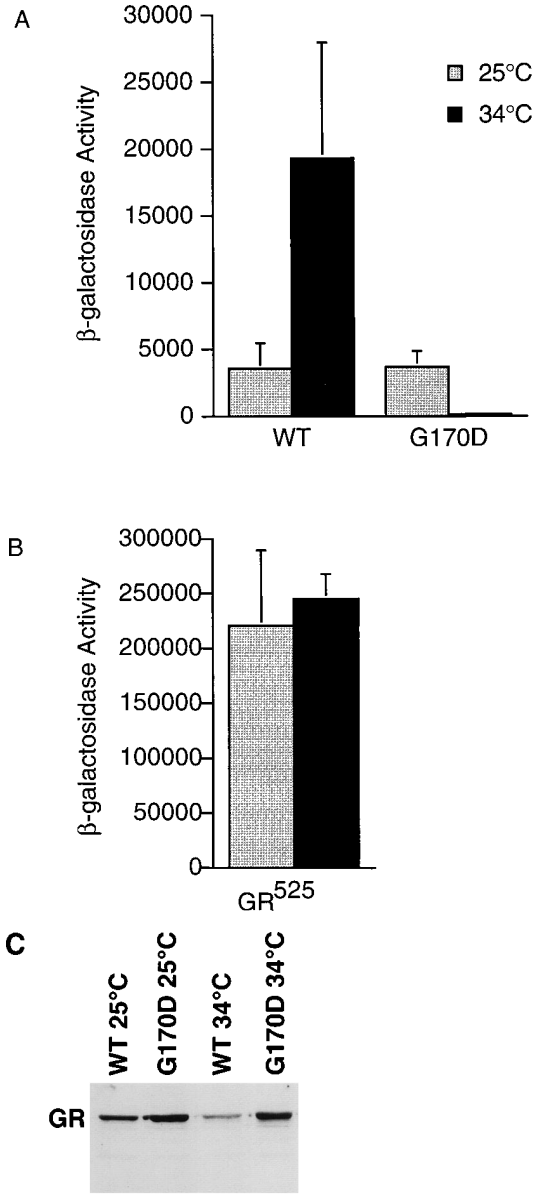

FIG. 5. Requirement for Hsp90 function after GR synthesis and maturation. Wild-type and G170D mutant cells were transformed with the galactose-inducible GR expression plasmid, p2HGAL/GR/CYC, or with a plasmid containing a mutant GR (GR^{525}) which has Hsp90-independent, constitutive activity. The cells were also transformed with the *lacZ* reporter plasmid, pSX26.1. Mid-log-phase cultures were transferred from raffinose to galactose medium and incubated at 25°C for 16 h to induce synthesis of GR. The cells were then transferred to glucose medium containing 10 μM DOC to stop further synthesis of GR and activate preformed aporeceptors. This medium was preequilibrated at 34°C to inactivate the G170D protein or at 25°C. WT, wild type. (A) Assessment of GR activity in wild-type and G170D cells and (B) assessment of GR^{525} activity in G170D cells. β-Galactosidase activities were normalized to the protein content of the lysate. The data shown are the means and standard deviations (error bars) of three independent experiments. (C) Assessment of GR accumulation. Total cellular proteins were separated on an SDS–7.5% polyacrylamide gel and transferred to an Immobilon-P membrane. Equal sample loading was confirmed by Coomassie blue staining (not shown), and the blot was then reacted with an antibody specific for GR. WT, wild type.

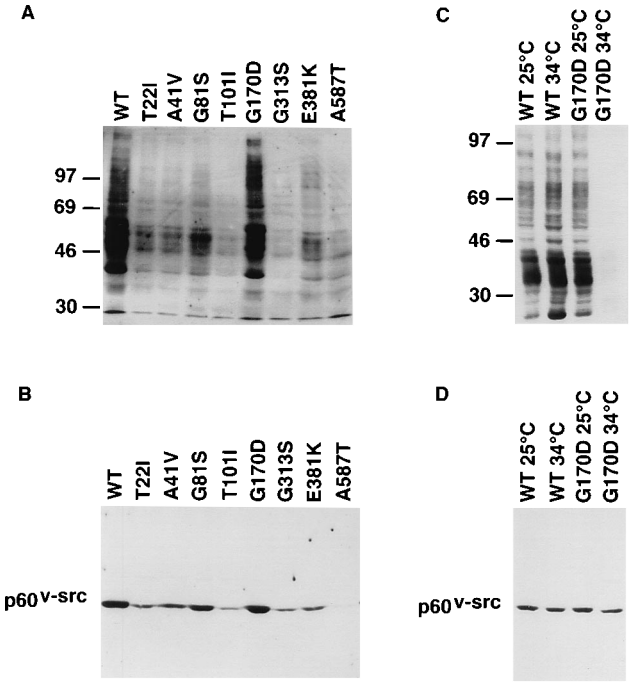

FIG. 6. $pp60^{v\text{-}src}$ activity and accumulation in the *hsp82* mutants. (A) Tyrosine phosphorylation mediated by $pp60^{v\text{-}src}$ in the *hsp82* mutants at 25°C. Wild-type (WT) and *hsp82* mutant strains were transformed with the galactose-inducible $pp60^{v\text{-}src}$ expression plasmid Y316v-src. The cells were grown to mid-log phase and transferred to inductive medium for 6 h. Total cellular proteins were separated on SDS–7.5% polyacrylamide gels and transferred to Immobilon-P membranes. Equal sample loading was confirmed by Coomassie blue staining (not shown), and the blot was reacted with antibody specific for phosphotyrosine. The numbers at the left of the gel indicate sizes in kilodaltons. (B) $pp60^{v\text{-}src}$ accumulation. An identical blot was reacted with an anti-v-*src* antibody to assess $pp60^{v\text{-}src}$ accumulation. (C) $pp60^{v\text{-}src}$ activity in the G170D mutant at 34°C. Wild-type and G170D mutant cells transformed with the $pp60^{v\text{-}src}$ expression plasmid were grown to mid log phase in raffinose. Half of the culture was transferred into galactose medium at 25°C, and the other half was transferred into galactose medium at 34°C. The cells were incubated for 6 h to induce $pp60^{v\text{-}src}$ expression. Plating experiments demonstrated that the viability of the G170D cells did not decline over the course of the experiment. Total cellular proteins were separated on SDS–7.5% polyacrylamide gels and transferred to Immobilon-P membranes. Equal sample loading was confirmed by Coomassie blue staining (not shown). The blot was reacted with an antibody specific for phosphotyrosine to assess $pp60^{v\text{-}src}$ activity. The numbers at the left indicate sizes in kilodaltons. (D) $pp60^{v\text{-}src}$ accumulation in the G170D mutant at 34°C. An identical blot was reacted with an anti-v-*src* antibody to assess $pp60^{v\text{-}src}$ accumulation.

protein than the A587T mutant but consistently had a lower level of kinase function.

Temperature shift experiments with the G170D mutant further distinguished the effects of Hsp90 on $pp60^{v\text{-}src}$ kinase activity and accumulation. At 25°C, G170D cells exhibited wild-type levels of $pp60^{v\text{-}src}$ activity and accumulation. When $pp60^{v\text{-}src}$ expression was induced after a shift to 34°C, $pp60^{v\text{-}src}$ kinase function was virtually eliminated but accumulation was only modestly reduced (Fig. 6C and D). Thus, Hsp90 plays a role in $pp60^{v\text{-}src}$ activation that is distinct from its role in promoting $pp60^{v\text{-}src}$ accumulation.

DISCUSSION

We have isolated eight Hsp90 point mutants that confer a ts phenotype on yeast cells lacking wild-type Hsp90. We have characterized these mutants in a manner not previously employed for heat shock protein mutants, distinguishing between those that are ts because they have general functional defects and fail to meet the increased demands that high temperatures place on heat shock protein function and those that are ts because they precipitously lose activity at high temperatures.

Rigorous classification of the mutants must await physical analysis of the purified proteins. Provisionally, we place seven of the eight mutants into the first category. In support of our classification, one of these mutants, T101I, has been purified and found to have the same thermal and guanidinium chloride-induced unfolding profile as the wild-type protein (9). The remaining mutant, G170D, behaves as a classic ts mutant with nearly wild-type function at 25°C and virtually no activity at temperatures above 34°C.

The unique properties of the G170D mutation provided the tool required to determine the function of Hsp90 in the mature GR aporeceptor complex in vivo. Previous work demonstrated that Hsp90 is required for GR to achieve a hormone-activable state in vivo (34). Once this state is achieved, however, the continued function of Hsp90 was not clear. On the one hand, some in vitro experiments suggested it has a negative role, keeping the DNA binding activity of GR repressed (31, 41, 43). On the other hand, some experiments demonstrated a positive role, maintaining hormone binding activity (7, 43). Our data do not exclude the possibility that Hsp90 has a dual role. They do, however, clearly demonstrate that Hsp90 is continuously required to maintain GR in a hormone-activable, transcriptionally competent state in vivo. Thus, the Hsp90 chaperone not only helps to establish the GR signal transduction pathway but is also an essential, positively acting, integral component of the pathway.

Both classes of mutations helped to clarify the role of Hsp90 in pp60$^{v\text{-}src}$ maturation. Reducing the concentration of wild-type Hsp90 dramatically reduces pp60$^{v\text{-}src}$ activity in yeast cells (56). However, because pp60$^{v\text{-}src}$ accumulation was also reduced, these experiments could not establish that Hsp90 had an effect on the activation state of the kinase. The difference in pp60$^{v\text{-}src}$ activity and accumulation in G313S and A587T cells at 25°C and the drastic loss of pp60$^{v\text{-}src}$ activity with only minor effects on accumulation in G170D cells at 34°C demonstrate that Hsp90 does affect the activation state of pp60$^{v\text{-}src}$. Thus, Hsp90 plays a direct role in establishing signaling by pp60$^{v\text{-}src}$ as well as by GR.

Perhaps the most puzzling feature of Hsp90 function is how the protein maintains such a high degree of specificity while interacting with such a diverse array of targets. Certain members of the steroid receptor family are highly dependent upon Hsp90, while others are not; certain protein kinases are highly dependent, while others are not. A priori, it might be that Hsp90's interactions with different classes of target protein are governed by different regions of the protein. However, we have found that eight single amino acid substitutions, originally selected for impaired function in yeast cells and located in different regions of the protein, all affect the ability of Hsp90 to functionally interact with two very different vertebrate target proteins, GR and pp60$^{v\text{-}src}$.

This correspondence in GR and pp60$^{v\text{-}src}$ phenotypes could be an artifact of having examined only a restricted class of mutations. Three lines of evidence argue that it is not. First, the mutations were broadly distributed throughout the protein, and a comparison with mutations obtained in other screens suggests that we are approaching saturation in terms of identifying essential elements of Hsp90 function. Three other screens, which included different selection criteria, different mutagens, and a different organism (*Drosophila melanogaster*), have identified 13 Hsp90 point mutations. Three (3, 23) are in exactly the same residues as mutations isolated in our screen, namely T101I, G170D, and G313N; two others (13) are in residues immediately adjacent to mutations isolated in our screen, namely E312K and S586F (with reference to the yeast *HSP82* sequence).

Second, our mutations were isolated on the basis of conferring ts growth in yeast cells, a selection criterion ostensibly independent of GR and pp60$^{v\text{-}src}$ activity. It was entirely plausible that some of these mutations would prove to have highly specific effects on as few as one critical yeast target protein. Yet, each of them affected the activity of two very different heterologous target proteins that have no known yeast homologs.

Third, the mutations we analyzed exhibit distinct phenotypes with respect to cell growth and cell morphology. They also have different effects on GR accumulation—some increase it, some decrease it—which must reflect differences in the mechanisms by which the mutations compromise Hsp90 function. Thus, the mutants represent a diverse collection, and the fact that they have corresponding effects on GR and pp60$^{v\text{-}src}$ activities is not simply an artifact of having examined only a restricted class of mutations.

As striking as the corresponding effects on GR and pp60$^{v\text{-}src}$ are, some differences were observed for the two target proteins. Although the activation of both GR and pp60$^{v\text{-}src}$ was blocked when the G170D mutant was shifted to high temperatures, accumulation was differentially affected: GR levels increased; pp60$^{v\text{-}src}$ levels decreased. It is unlikely that this difference in accumulation reflects a fundamental difference in the way these two target proteins interact with Hsp90. Rather, it is likely to reflect distinct intracellular fates after their interactions with Hsp90 have been compromised. A potentially more interesting example of an individual Hsp90 mutation exerting distinct effects on different target proteins was reported by Bohen and Yamamoto (3): the E431K mutation affects the activity of GR but not the activity of three other steroid receptors, estrogen receptors, PR, and mineralocorticoid receptors. This mutation may identify an Hsp90 function that is unique to GR. In light of our findings, however, it seems more likely that it identifies a residue in a region of common function where point mutations can specifically affect the interaction of Hsp90 with individual targets.

A key to understanding Hsp90 function lies in determining the function of other members of the target protein complex. Some members seem to be unique to particular classes of target protein, while others are apparently common to all (4, 8, 17–19, 21, 35, 39, 49, 50–52, 54). Hsp90 binds to its target proteins when associated with certain of these proteins (21, 39, 50), and at least one of them, Hsp70, is required for Hsp90 to bind GR and PR during in vitro reconstitution (19, 50). Some of our mutants may affect Hsp90-target protein interactions directly. Others may affect the target protein indirectly by altering Hsp90's interaction with one or more of these complex members. Indeed, preliminary analysis indicates that some of our mutants affect Hsp90's interaction with Sti1, the yeast homolog of p60. Strikingly, different Hsp90 mutants affect this interaction in different ways. In some, overexpression of Sti1 increases the maximal growth temperature; in others, it decreases the maximal growth temperature; in yet others, it has no effect (30).

The different effects of Sti1 on our collection of Hsp90 mutants provide yet another demonstration that this collection is diverse. Clearly, the mutants compromise Hsp90 function in mechanistically distinct ways. That all of these mutants exert negative affects on the activity of both GR and pp60$^{v\text{-}src}$ indicates that Hsp90 promotes signaling by these molecules through a common mechanism.

ACKNOWLEDGMENTS

We thank Keith Yamamoto for the *HSP82* sequencing primers and GR expression plasmids and David Morgan for v-*src* expression plas-

mids. We are grateful to Johannes Buchner for sharing unpublished data and for insightful comments on the manuscript. We also thank Bushra Khursheed for constructing the *hsp82* and *hsc82* disruption mutants, Laura Arwood for constructing the p2A/GRGZ GR expression plasmid, and Gina Kim for her excellent technical assistance. Finally, we thank Yoko Kimura, Jane Chang, Lin Yue, Eric Schirmer, and John Glover for their thoughtful comments on the manuscript.

This work was supported by the Howard Hughes Medical Institute and by National Institutes of Health fellowship GM14745-02 (D.F.N.).

REFERENCES

1. **Bardwell, J. C. A., and E. A. Craig.** 1988. Ancient heat shock gene is dispensable. J. Bacteriol. **170:**2977–2983.
2. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:**345–346.
3. **Bohen, S. P., and K. R. Yamamoto.** 1993. Isolation of Hsp90 mutants by screening for decreased steroid receptor function. Proc. Natl. Acad. Sci. USA **90:**11424–11428.
4. **Bohen, S. P., and K. R. Yamamoto.** 1994. Modulation of steroid receptor signal transduction by heat shock proteins, p. 313–334. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
5. **Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist.** 1989. Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. **9:**3919–3930.
6. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:**17–24.
7. **Bresnick, E. H., F. C. Dalman, E. R. Sanchez, and W. B. Pratt.** 1989. Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. J. Biol. Chem. **264:**4992–4997.
8. **Brugge, J. S.** 1986. Interaction of the Rous sarcoma virus protein pp60^{src} with the cellular proteins pp50 and pp90. Curr. Top. Microbiol. Immunol. **123:**1–22.
9. **Buchner, J., I. Meyer, U. Jakob, D. F. Nathan, and S. Lindquist.** Unpublished data.
10. **Catelli, M. G., N. Binart, I. Jung-Testas, J. M. Renoir, E. E. Baulieu, J. R. Feramisco, and W. J. Welch.** 1985. The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. EMBO J. **4:**3131–3135.
11. **Chang, H.-C. J., and S. Lindquist.** 1994. Conservation of Hsp90 macromolecular complexes in *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**24983–24988.
12. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.
13. **Cutforth, T., and G. M. Rubin.** 1994. Mutations in *Hsp83* and *cdc37* impair signaling by the Sevenless receptor tyrosine kinase in Drosophila. Cell **77:** 1027–1036.
14. **Dalman, F. C., R. J. Koenig, G. H. Perdew, E. Massa, and W. B. Pratt.** 1990. In contrast to the glucocorticoid receptor, the thyroid hormone receptor is translated in the DNA binding state and is not associated with hsp90. J. Biol. Chem. **265:**3615–3618.
15. **Dalman, F. C., L. J. Sturzenbecker, A. A. Levin, D. A. Lucas, G. H. Perdew, M. Petkovitch, P. Chambon, J. F. Grippo, and W. B. Pratt.** 1991. Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking" complexes with hsp90. Biochemistry **30:**5605–5608.
16. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
17. **Hutchison, K. A., B. K. Brott, J. H. De Leon, G. H. Perdew, R. Jove, and W. B. Pratt.** 1992. Reconstitution of the multiprotein complex of pp60^{src}, Hsp90, and p50 in a cell free system. J. Biol. Chem. **267:**2902–2908.
18. **Hutchison, K. A., M. J. Czar, L. C. Scherrer, and W. B. Pratt.** 1992. Monovalent cation selectivity for ATP-dependent association of the glucocorticoid receptor with Hsp70 and Hsp90. J. Biol. Chem. **267:**14047–14053.
19. **Hutchison, K. A., K. D. Dittmar, M. J. Czar, and W. B. Pratt.** 1994. Proof that Hsp70 is required for assembly of the glucocorticoid receptor into a heterocomplex with Hsp90. J. Biol. Chem. **269:**5043–5049.
20. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
21. **Johnson, J. L., and D. O. Toft.** 1994. A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. J. Biol. Chem. **269:**24989–24993.
22. **Johnston, M., and R. W. Davis.** 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**1440–1448.
23. **Kimura, Y., S. Matsumoto, and I. Yahara.** 1994. Temperature-sensitive mutants of hsp82 of the budding yeast, Saccharomyces cerevisiae. Mol. Gen. Genet. **242:**517–527.
24. **Landman, O. E., H. T. Bausum, and T. S. Matney.** 1962. Temperature-gradient plates for growth of microorganisms. J. Bacteriol. **83:**463–469.
25. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
26. **McCusker, J. H., and R. W. Davis.** 1991. The use of proline as a nitrogen source causes hypersensitivity to, and allows more economical use of 5FOA in *Saccharomyces cerevisiae*. Yeast **7:**607–608.
27. **Minami, Y., Y. Kimura, H. Kawasaki, K. Suzuki, and I. Yahara.** 1994. The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function in vivo. Mol. Cell. Biol. **14:**1459–1464.
28. **Miyata, Y., and I. Yahara.** 1992. The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. J. Biol. Chem. **267:**7042–7047.
29. **Murphy, S. M., M. Bergman, and D. O. Morgan.** 1993. Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**5290–5300.
30. **Nathan, D. F., H.-C. J. Chang, and S. Lindquist.** Unpublished data.
31. **Nemoto, T., G. G. F. Mason, A. Wilhelmsson, S. Cuthill, J. Hapgood, J.-A. Gustafsson, and L. Poellinger.** 1990. Activation of the dioxin and glucocorticoid receptors to a DNA binding state under cell-free conditions. J. Biol. Chem. **265:**2269–2277.
32. **Pakula, A. A., and R. T. Sauer.** 1989. Genetic analysis of protein stability and function. Annu. Rev. Genet. **23:**289–310.
33. **Parsell, D. A., and S. Lindquist.** 1994. Heat shock proteins and stress tolerance, p. 457–494. *In* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Picard, D., B. Khursheed, M. J. Garabedian, M. G. Fortin, S. Lindquist, and K. R. Yamamoto.** 1990. Reduced levels of hsp90 compromise steroid receptor action *in vivo*. Nature (London) **348:**166–168.
35. **Pratt, W. B.** 1993. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J. Biol. Chem. **268:** 21455–21458.
36. **Redeuilh, G., B. Moncharmont, C. Secco, and E.-E. Baulieu.** 1987. Subunit composition of the molybdate-stabilized "8-9 S" nontransformed estradiol receptor purified from calf uterus. J. Biol. Chem. **262:**6969–6975.
37. **Rose, D. W., R. E. H. Wettenhall, W. Kudlicki, G. Kramer, and B. Hardesty.** 1987. The 90-kilodalton peptide of the heme-regulated eIF-2α kinase has sequence similarity with the 90-kilodalton heat shock protein. Biochemistry **26:**6583–6587.
38. **Rose, M. D., F. Winston, and P. Hieter.** 1989. Laboratory course manual for methods in yeast genetics, p. 139. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sanchez, E. R., L. E. Faber, W. J. Henzel, and W. B. Pratt.** 1990. The 56-59-kilodalton protein identified in untransformed steroid receptor complexes is a unique protein that exists in cytosol in a complex with both the 70- and 90-kilodalton heat shock proteins. Biochemistry **29:**5145–5152.
40. **Sanchez, E. R., J.-L. Hu, S. Zhong, P. Shen, M. J. Greene, and P. R. Housley.** 1994. Potentiation of glucocorticoid receptor-mediated gene expression by heat and chemical shock. Mol. Endocrinol. **8:**408–421.
41. **Sanchez, E. R., S. Meshinchi, W. Tienrungroj, M. J. Schlesinger, D. O. Toft, and W. B. Pratt.** 1987. Relationship of the 90-kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. J. Biol. Chem. **262:**6986–6991.
42. **Sanchez, E. R., D. O. Toft, M. J. Schlesinger, and W. B. Pratt.** 1985. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. **260:**12398–12401.
43. **Scherrer, L. C., F. C. Dalman, E. Massa, S. Meshinchi, and W. B. Pratt.** 1990. Structural and functional reconstitution of the glucocorticoid receptor-Hsp90 complex. J. Biol. Chem. **265:**21397–21400.
44. **Schuh, S., W. Yonemoto, J. Brugge, V. J. Bauer, R. M. Riehl, W. P. Sullivan, and D. O. Toft.** 1985. A 90,000-dalton binding protein common to both steroid receptors and the Rous sarcoma virus transforming protein, pp60$^{v\text{-}src}$. J. Biol. Chem. **260:**14292–14296.
45. **Schumacher, R. J., R. Hurst, W. P. Sullivan, N. J. McMahon, D. O. Toft, and R. L. Matts.** 1994. ATP-dependent chaperoning activity of reticulocyte lysate. J. Biol. Chem. **269:**9493–9499.
46. **Shue, G., and D. S. Kohtz.** 1994. Structural and functional aspects of basic helix-loop-helix protein folding by heat-shock protein 90. J. Biol. Chem. **269:**2707–2711.
47. **Sikorski, R. S., and J. D. Boeke.** 1991. *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. Methods Enzymol. **194:**302–318.
48. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
49. **Smith, D. F.** 1993. Dynamics of heat shock protein 90-progesterone receptor

binding and the disactivation loop model for steroid receptor complexes. Mol. Endocrinol. **7:**1418–1429.
50. **Smith, D. F., B. A. Stensgard, W. J. Welch, and D. O. Toft.** 1992. Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. J. Biol. Chem. **267:**1350–1356.
51. **Smith, D. F., and D. O. Toft.** 1993. Steroid receptors and their associated proteins. Mol. Endocrinol. **7:**4–11.
52. **Stancato, L. F., Y.-H. Chow, K. A. Hutchison, G. H. Perdew, R. Jove, and W. B. Pratt.** 1993. Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. J. Biol. Chem. **268:**21711–21716.
53. **Taulien, J., D. F. Nathan, and S. Lindquist.** Unpublished data.
54. **Wartmann, M., and R. J. Davis.** 1994. The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. J. Biol. Chem. **269:**6695–6701.
55. **Wiech, H., J. Buchner, R. Zimmermann, and U. Jakob.** 1992. Hsp90 chaperones protein folding *in vitro*. Nature (London) **358:**169–170.
56. **Xu, Y., and S. Lindquist.** 1993. Heat-shock protein hsp90 governs the activity of $pp60^{v\text{-}src}$ kinase. Proc. Natl. Acad. Sci. USA **90:**7074–7078.