Hsp90 Regulates Androgen Receptor Hormone Binding Affinity in Vivo*

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The regulation of human androgen receptor (AR) by the molecular chaperone Hsp90 was investigated using the yeast Saccharomyces cerevisiae as a model system. These studies were performed in strains expressing a conditional temperature-sensitive mutant allele of the hsp82 gene, which encodes Hsp90 protein. At the restrictive temperature in the mutant, there is a decrease in hormone-dependent transactivation by the AR, although steady state levels of AR protein are unchanged. Quantitative hormone binding studies at the permissive temperature revealed the presence of both high affinity and low affinity hormone binding states. At the restrictive temperature in the hsp82 mutant, the high affinity state was abolished, and only the low affinity state was observed. The change in hormone binding affinity was further investigated by a competition assay with the anti-androgen hydroxyflutamide. Under permissive conditions, hydroxyflutamide competes poorly for the synthetic androgen R1881, but under restrictive conditions in the hsp82 mutant strain, hydroxyflutamide was shown to be a potent competitive inhibitor. Our findings indicate that Hsp90 participates in the activation process by maintaining apoAR in a high affinity ligand binding conformation which is important for efficient response to hormone.

Steroid hormones bind to intracellular receptors and transform them into active transcription factors (see Ref. 1 for review). Prior to ligand binding, the inactive apo-receptors are maintained in a heterocomplex with several molecular chaperones. Among these are Hsp90 and Hsp70 as well as several other proteins, some that have been characterized as peptidyl-prolyl isomerases and some that remain relatively uncharacterized. Among the latter are three proteins called p60 (2), p48 (3, 4), and p23 (5). It appears that all of these proteins are bound to steroid hormone receptors indirectly via Hsp90 (see Refs. 6–8 for reviews).

Hsp90 is required for high affinity ligand binding to several steroid hormone receptors, but with different requirements. In the case of glucocorticoid receptor (GR), Hsp90 is required for high affinity ligand binding, and in the absence of the chaperone the affinity for hormone is reduced by 100-fold (9–11). High affinity hormone binding to mineralocorticoid (MR) and dioxin receptors similarly require Hsp90 (12, 13). Progesterone receptor (PR) appears to behave differently, however, since high affinity hormone binding occurs in the absence of Hsp90 at 4°C, although not at 37°C (4).

The AR displays similarity with PR since high affinity hormone binding (with dissociation constants (Kd) in the low nM range) was observed in vitro in the absence of Hsp90. Studies by Nemoto et al. (14) compared the affinity of hormone for AR after in vitro expression in rabbit reticulocyte lysates and in vivo expression in Escherichia coli. In these experiments, the in vitro expression results in association of AR with Hsp90, while expression in E. coli does not. Nemoto et al. (14) observed small differences in the affinity of hormone for AR in these two systems, but concluded that these were not the consequence of Hsp90 binding.

We have utilized the yeast system as an in vivo environment in which to study the role of Hsp90 in AR regulation. Previous studies revealed that AR function is hormone dependent in yeast cells, suggesting conservation in the regulatory machinery that controls activation (15, 16). Furthermore, yeast strains expressing reduced amounts or mutant forms of Hsp90 are defective for transactivation by estrogen, GR, MR, and PR (11, 17–19). Using yeast strains expressing a mutant form of a gene encoding Hsp90, we tested how Hsp90 loss of function affected AR activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains used in this study are described in Table I. Yeast cultures were grown under standard conditions in medium containing 0.67% yeast nitrogen base (Difco), 2% dextrose, and appropriate amino acid supplements. Transformation of plasmids into yeast cells was performed according to method described by Gietz et al. (20). Plasmids that express the AR (pARU and pARH) were derived from pG1-hAR (16). pARU was constructed by inserting a blunt-ended URA3 gene fragment into EcoRV-linearized pG1-hAR (within the TRP1 gene). pARH was constructed by inserting a blunt-ended HIS3 gene fragment into EcoRV-linearized pG1-hAR. pPGKareLacZC contains the lacZ gene under control of three cis-acting androgen response elements as described by Purvis et al. (15).

β-Galactosidase Activity Assay—Yeast cultures were grown to the log phase and incubated at 25 or 37°C for 30 min prior to addition of dihydrotestosterone (DHT) for 1 h. Assays for β-galactosidase activity in whole cell extracts were as described previously (16).

Hormone Binding Assays—Yeast cells were grown to early log phase (A590 = 0.2) and preincubated at 25 or 37°C in 1-ml aliquots for 30 min. H-R1881 (New England Nuclear) was added to the cultures (usually diluted 1:20 or 1:50 with unlabeled R1881 in ethanol) in the presence and absence of a 167-fold excess of DHT. The cells were further incubated for 90 min, washed three times with 1 ml of water, and counted in 5 ml of scintillation fluid in a scintillation counter. Specific binding was determined by subtracting the counts/min from samples incubated with DHT (giving background counts/min) from samples incubated without DHT. The background counts/min were approximately 5% of total counts.

Competition assays were performed by incubating yeast cells with

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§ The abbreviations used are: GR, glucocorticoid receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; DHT, dihydrotestosterone; AR, androgen receptor; NTA, nitritolriacetic acid.
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Table I
Yeast strains used

| Strain   | Genotype                                      | Source                      |
|----------|-----------------------------------------------|-----------------------------|
| W3031b   | ada2-1 leu2-3, 112 his3-11, 15 trp1-1 ura3-1 can1-100 | Thomas and Rothstein (37)  |
| ACY44    | ada2 leu2 his3 trp1 ura3 can1 pG1-hAR pPGKareLacZI | Caplan et al. (16)          |
| GPDHsp82 | ada2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) pGPDHsp82 | Chang and Lindquist (21)    |
| GPDHsp82F | ada2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) pGPDHsp82 | This study                  |
| ACY88    | GPDHsp82 with pARU                            | This study                  |
| ACY89    | GPDHsp82F with pARU                           | This study                  |
| P82a     | ada2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) pGPDHsp82 | This study                  |
| G170Da   | ada2 leu2 his3 trp1 ura3 can1 (hsc82::LEU2) pGPDHsp82 | This study                  |
| ACY98    | P82a with pARH                                 | This study                  |
| ACY99    | G170Da with pARH                              | This study                  |
| ACY100   | P82a with pARH & pPGKareLacZC                 | This study                  |
| ACY101   | G170Da with pARH & pPGKareLacZC               | This study                  |

All strains are derived from W303.

100 nm $^{3}$H-R1881 and 0–500 μM hydroxyflutamide (Scherring-Plough, stored in ethanol). The cells were washed three times in water and counted in 5 ml of scintillant. Counts/min for each sample are presented as a percentage of the counts/min from cells incubated without hydroxyflutamide. All results are the mean of three to five independent experiments.

Immuno precipitation of AR and Affinity Isolation of Hsp90—For immunoprecipitation of AR, cells were grown to the log phase, harvested by centrifugation, and washed with buffer A (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 15 mM MgCl$_2$, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg each of leupeptin, pepstatin, aprotinin, and chymostatin). The cells were pelleted, resuspended in 0.8 ml of lysis buffer, and broken in the presence of 1.5 g of 0.4-mm glass beads (w/v), and crude lysates were cleared as described above. 500 μl of protein extract (3 mg/ml) were incubated with 100 μl of Ni-NTA-agarose beads (Qiagen, 50% slurry in lysis buffer) for 1 h on a nutator. The beads were collected at 500 × g in a microcentrifuge and washed twice in lysis buffer plus 5 mM imidazole and twice in lysis buffer plus 10 mM imidazole. The beads were incubated with 1 ml of elution buffer (lysis buffer plus 150 mM imidazole) at 4°C for 10 min. Eluted proteins were precipitated with 10% trichloroacetic acid in the presence of 8 μg/ml insulin β-chain as carrier (21).

Miscellaneous—SDS-polyacrylamide gels were prepared and run according to standard procedures. Western blots were performed as described previously (16). Antisera to AR was prepared using a mouse AR-glutathione S-transferase fusion protein (containing residues 133–334 of AR) in rabbits. Antisera were prepared by Lampire Biological Laboratories, Pipersville, PA. Antisera to yeast Hsp90 were described previously by Dey et al. (22).

RESULTS

Interaction of Hsp90 with the Androgen Receptor—The experimental basis for studying steroid hormone receptors in yeast cells relies on the conservation of the cellular machinery that controls receptor activation. This criterion is satisfied to some extent by the structural similarity in Hsp90 chaperone complexes found in yeast and higher eukaryotes (21). Furthermore, complexes between GR and yeast Hsp90 have been characterized (11, 18, 21). For the present study, we confirmed that AR could also form a specific complex with Hsp90 in wild type yeast cells.

In the experiment shown in Fig. 1A, the AR was immunoprecipitated from wild type yeast whole cell extracts, and the presence of Hsp90 was detected after Western blot analysis. Co-immunoprecipitation of Hsp90 with AR was considered specific since it occurred neither with preimmune sera (Fig. 1A, lane 2) nor with immune sera incubated with extracts from cells not expressing AR (Fig. 1A, lane 1). Similarly, AR protein specifically co-isolated with Hsp90 after affinity chromatography (21). For the experiment shown in Fig. 1B, human AR was co-expressed with an His$_6$-tagged Hsp90 protein (His$_6$-Hsp90) in yeast cells. Extracts from these cells were incubated with Ni-NTA-agarose beads to affinity purify the His$_6$-Hsp90 and His$_6$-Hsp90 binding proteins. Proteins eluted from the Ni-NTA-agarose beads were resolved by SDS-PAGE and ARs that co-isolated with His$_6$-Hsp90 were detected by Western blot analysis (Fig. 1B, lane 3). In a similar experiment with non-tagged wild type Hsp90, neither Hsp90 nor AR were bound to Ni-NTA-agarose beads (Fig. 1B, lane 4).

These data confirm that the interaction between Hsp90 and AR occurs in yeast, as it does in higher eukaryotes (14, 23, 24). We next tested how loss of Hsp90 function affected AR activation using yeast strains expressing a mutant hsp82 gene.

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hsp82 Mutant Strains—Previous studies with Saccharomyces cerevisiae established that Hsp90 is important for hormone dependent transactivation by estrogen, GR, MR, and PR (11, 17–19). For the present study, a yeast strain expressing a mutant version of the hsp82 gene was used to study the role of Hsp90 protein in AR regulation. This mutant, first characterized by Nathan and Lindquist (19), is a substitution of glycine 170 for aspartate in Hsp90. Both endogenous genes encoding Hsp90 proteins (HSC82 and HSP82) were deleted from this strain, and the mutant hsp82 gene was transcribed constitutively from the glyceraldehyde-3-phosphate dehydrogenase gene promoter, present on a low copy number plasmid. The mutant strain is temperature-sensitive for growth and is inviable above 33 °C. An isogenic wild type strain, viable at all temperatures tested, was used as a control for the experiments shown below. This wild type strain is also deleted for both endogenous genes encoding Hsp90 proteins and expresses the wild type HSP82 also from a low copy number plasmid (19).

To determine the effect of Hsp90 loss of function on AR activation, both wild type and mutant (hsp82) strains were transformed with two plasmids: one that constitutively expressed the human AR and another that expressed the E. coli lacZ gene under control of the AR. lacZ gene expression was hormone dependent in these strains, and induction was determined by measuring soluble β-galactosidase activity in whole cell extracts.

Growing cultures of wild type and hsp82 cells were incubated at temperatures that were permissive (25 °C) or restrictive (37 °C) for the hsp82 strain in the presence or absence of 100 nM DHT for 1 h. At 25 °C, similar levels of β-galactosidase were observed in both strains after hormone addition (Fig. 2A). Despite this similarity, the induction ratio was 2.4-fold greater in the wild type (22-fold above background) compared with the mutant (9-fold above background). This occurred because uninduced β-galactosidase levels in the mutant were consistently 2-fold higher than in the wild type. This increase was AR independent since it was observed even in the absence of the AR in these strains (data not shown). At 37 °C, induction in the mutant was reduced to less than 3-fold (Fig. 2B), compared with a mean 10-fold induction in the wild type. This was partly due to increased background although total β-galactosidase levels were still approximately 2-fold less in the mutant than in the wild type.

The induction difference between wild type and hsp82 mutant strains was accentuated at subsaturating DHT concentrations; at 10 nM DHT for example, lacZ gene induction was 9-fold in the wild type, but less than 2-fold in the mutant (Fig. 2C). Thus, in comparison to the wild type, the mutant strain displayed a 3–5-fold decrease in hormone dependent activation at the restrictive temperature, depending on hormone level.

These data suggested that the AR had a decreased ability to respond to hormone upon Hsp90 loss of function. This was not, however, a result of decreasing amounts of receptor, since Western blot analysis revealed similar AR protein levels in both strains (Fig. 3). The apparent decrease in full-length AR in the wild strain was observed in other experiments; similar differences in protein turnover have been reported for GR in these strains (19).

Ligand Binding to the Androgen Receptor in Wild Type and hsp82 Mutant Strains—As described above, DHT fails to properly activate AR in the hsp82 mutant, despite there being little change in receptor protein levels. One possible explanation for these data was that Hsp90 regulated ligand binding to the receptor. This was investigated by calculating the hormone binding affinity of AR using an in vivo ligand binding assay. In this experiment, growing yeast cultures were treated with [3H]R1881 (a synthetic androgen) over a range of hormone concentrations at 25 or 37 °C after a 30-min preincubation at these temperatures. These studies provided a basis for direct comparison of the binding characteristics in wild type and mutant strains. Binding of labeled androgen to these cells was dependent on the presence of the AR, since in its absence there was negligible specific binding.

The binding data are complex since there appeared to be two...
binding states that differed in their affinity for the hormone. As revealed by Scatchard analysis (Fig. 4, B–E), the high affinity state was apparent in a linear plot between 2.5 nM and 50 nM R1881, and the low affinity state between 50 nM and 500 nM R1881 (Fig. 4, B–E, dotted lines). This was also suggested by direct inspection of the primary data, since hormone binding appeared to level off at 50 nM R1881 (except the mutant at 37°C; see Fig. 4A, inset), but continued to rise approximately 2-fold upon a further 10-fold increase in hormone concentra-
tion. The existence of both high and low affinity states has been observed previously with retinoic acid receptors expressed in yeast cells (30).

In Fig. 4, B–E, the high affinity state is evident in the wild type at both temperatures (with apparent K_d values at 0.8 nM and 1.0 nM at 25 and 37°C, respectively) but in the mutant only at 25°C (apparent K_d = 2.1 nM). When assayed at 37°C, the hsp82 mutant failed to display the high affinity binding state (Fig. 4E) and instead, showed only the low affinity state (ap-
parent $K_d = 46.8 \pm 11.2$ nM for all samples). In these yeast strains, therefore, hormone binds to the AR with a similar affinity to that observed by other investigators in this (25) and in other systems (14, 26–28), but in a manner that is dependent on Hsp90 function.

The loss of high affinity R1881 binding sites in the hsp82 mutant strain was further investigated by competition studies. These were performed using the anti-androgen hydroxyflutamide, which has a relative binding affinity for AR that is 50-fold less than R1881 (29). When tested at micromolar concentrations against the AR in yeast cells, hydroxyflutamide was an agonist in the absence of DHT and an antagonist in the presence of DHT, consistent with previous studies by Wong et al., (28).

In the wild type yeast strain, there was little competition for $^3$H-R1881 over a 0–500-fold excess of cold hydroxyflutamide, either at 25 or 37 °C. In the hsp82 mutant, however, significant competition was observed at the restrictive temperature of 37 °C, but not at the permissive temperature of 25 °C (Fig. 5). Only a 5-fold excess of hydroxyflutamide was required to reduce the binding of $^3$H-R1881 to 50% of the starting level, whereas a 500-fold excess reduced these levels by only 25–30% in the wild type at either temperature or the mutant at 25 °C.

DISCUSSION

The results presented in this report indicate that Hsp90 maintains the AR in a high affinity hormone binding conformation. Upon Hsp90 loss of function, the AR loses the high affinity state, and as an ultimate consequence, is less active as a transcription factor in the presence of hormone (Fig. 2). Even in wild type cells, however, there exist two pools of receptor, one with high hormone affinity and one of low affinity (Fig. 4).

While the nature of the low affinity state is unclear, its existence in all cells may reflect an equilibrium between receptors that are poised for activation, by having high hormone affinity, and those that are not (36). A model based upon these data is presented in Fig. 6. In this model, the apo-receptor is shown to be in equilibrium between low (L) and high (H) affinity states. A third state is shown corresponding to the active (A) receptor.

In the model, Hsp90 pushes the equilibrium toward the high affinity hormone binding conformation. In this state, the binding of hormone leads to further structural changes that contribute to receptor activation. Upon Hsp90 loss of function (at 37 °C in the mutant), the low affinity state is favored, and in the presence of hormone, the receptor is less able to adopt the active state. Although apparent even in wild type cells, we cannot conclude that the low affinity state measured as a consequence of Hsp90 loss of function is identical to that which occurs in the presence of functional Hsp90.

While the nature of the conformational changes in AR are unknown, there are two possible explanations that take into account a role for Hsp90 in maintaining the high affinity state. The first is that the hormone binding domain tends toward instability in the absence of Hsp90. The role of Hsp90 might then be to stabilize a conformation in AR that has a higher affinity for hormone. In this sense, Hsp90 functions as a molecular chaperone by stabilizing an otherwise unstable conformation (33).

Alternatively, the low affinity hormone binding state may reflect a kinetically trapped folding intermediate that requires not only the action of Hsp90 but also other proteins that associate with it. This could conceivably involve the peptidylprolyl isomerases known to complex with Hsp90 and steroid hormone receptors (6, 8), since these ubiquitous enzymes are well characterized in their role as catalysts of protein folding (31). In this manner, Hsp90 differs from the Hsp70 and Hsp60 (groE) chaperones.
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erone machines since it integrates an enzymatic activity. The role of Hsp90 might thus be likened to a stage for the action of other chaperones, or in this case, enzymes. Indeed, it was recently demonstrated that high affinity hormone binding to PR requires the Hsp90-peptidylprolyl isomerase-p23 complex, but not the Hsp90/Hsp70/p60 complex (34).

While the results from our genetic approach strongly support a role for Hsp90 in maintaining the high affinity hormone binding state, they clearly contrast with previous studies. For example, Ohara-Nemoto et al. (32) and Nemoto et al. (14) observed only slight changes in AR hormone binding affinity in the presence or absence of Hsp90, and Xie et al. (26) calculated a $K_0$ of 8 nM for R1881 binding to purified AR after refolding denatured protein (compared with $K_0$ = 5 nM in cell extracts). An important experimental difference rests with our use of yeast as an intracellular environment in contrast to the use of purified components or cell extracts in the studies described above. A more compelling interpretation of these differences, however, is that the AR may display a conditional Hsp90 dependence for high affinity hormone binding. As previously noted, AR displays some similarity with PR since at low temperatures both receptors bind ligand with high affinity in the absence of Hsp90. At 37°C, however, PR requires Hsp90 to maintain the high affinity steroid binding conformation (4). If such temperature dependence also applied to AR, then no significant change in hormone binding affinity may be expected at 4°C, but would be apparent at 37°C as was shown in Fig. 4. The hormone binding studies of Ohara-Nemoto et al. (32), Nemoto et al. (14), and Xie et al. (26) were all performed at low temperature.

Interestingly, such temperature dependence does not appear to be a general phenomenon among steroid hormone receptors. When assayed at low temperatures in the absence of Hsp90, there is a 100-fold decrease in the hormone binding affinity of GR, and a 1000-fold change for MR compared with studies performed in the presence of Hsp90 (10, 13). Such differences suggest that Hsp90 has specific receptor-dependent functions. In this context, Bohen and Yamamoto (18) have characterized specific mutations in Hsp90 that affect receptors differentially. For example, substitution of glutamate 431 by lysine, has little effect on the function of estrogen, MR, or PR, but profoundly affects GR. Different receptors, therefore, have specialized requirements, although whether these reflect differences in degree or in other specific needs is complex and compounded by the varied cast of Hsp90 binding proteins.

Hsp90 binds to several proteins known to be involved in signal transduction, most of which are transcription factors or protein kinases (35). What each of these has in common is that the cell must maintain them in an "off" state prior to activation. Whether the Hsp90 chaperone machine has a general role in maintaining repression of these factors or in facilitating ligand-dependent activation may yet prove to be a fundamental principle of signal transduction.

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