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Control of estrogen receptor ligand binding by Hsp90

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Abstract

The molecular chaperone Hsp90 interacts with unliganded steroid hormone receptors and regulates their activity. We have analyzed the function of yeast and mammalian Hsp90 in regulating the ability of the human estrogen receptor (ER) to bind ligands in vivo and in vitro. Using the yeast system, we show that the ER expressed in several different *hsp82* mutant strains binds reduced amounts of the synthetic estrogen diethylstilbestrol compared to the wild type. This defect in hormone binding occurs without any significant change in the steady state levels of ER protein. To analyze the role of mammalian Hsp90, we synthesized the human ER in rabbit reticulocyte lysates containing geldanamycin, an Hsp90 inhibitor. At low concentrations of geldanamycin we observed reduced levels of hormone binding by the ER. At higher concentrations, we found reduced synthesis of the receptor. These data indicate that Hsp90 functions to maintain the ER in a high affinity hormone-binding conformation. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Steroid; Receptor; Hormone binding; Estrogen receptor; Hsp90; Molecular chaperone; Geldanamycin

1. Introduction

Hsp90 molecular chaperones regulate signal transduction by steroid hormone receptors and protein kinases. In the case of steroid hormone receptors, Hsp90 associates with the unliganded forms of the androgen receptor (AR; [1]), ER [2], glucocorticoid receptor (GR; [3]), mineralocorticoid receptor (MR; [4]) and the progesterone receptor (PR; [5–7]). Hormone binding stimulates Hsp90 dissociation, and this leads to receptor dimerization, interaction with co-activators, DNA binding and target gene activation (for review see [8]). Hsp90 function appears to be related to receptor folding, especially of the hormone binding domains. For example, it has been found from experiments performed in vitro that Hsp90 is essential for hormone binding to the GR under all conditions [9], although it is required for high affinity hormone binding to the PR only at high temperatures [10]. In the case of the AR, Hsp90 is important for hormone binding in a yeast model system, although it is sufficiently stable in vitro to support high-affinity hormone binding in the absence of Hsp90 [5,11]. From these examples it is clear that Hsp90 controls ligand binding but to different extents, depending on the receptor and on the environmental conditions.

Studies on the ER have provided an even more complex picture. Results from experiments using yeast as a model system have demonstrated that Hsp90 is required for efficient activation, although in a less stringent manner than for the GR [12]. And, although Hsp90 forms complexes with unliganded ER, there is evidence from several lines of enquiry to suggest that it does not influence ligand binding. First, purified ER or ER expressed in *E. coli* has an affinity for hormone that is very similar to that found inside animal cells [13,14]. Second, mutants of Hsp90 that do affect hormone binding to MR have no effect on ligand binding to the ER in baculovirus-infected insect cells [16]. This suggests that the ER is stable under conditions where

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the MR is unstable and requires the action of Hsp90. Third, chimeric forms of the ER can stimulate target gene expression in a ligand dependent manner without apparently interacting with Hsp90 [17]. There is also some evidence that Hsp90 functions in ER activation at a stage that is downstream of hormone binding. In these studies [18], Hsp90 was found to affect binding of the ER to DNA and to be able to dissociate ER:DNA complexes.

An alternative view is derived from studies where Hsp90 itself was inhibited by treatment of cells with geldanamycin, followed by subsequent analysis of hormone binding by the ER. The results of these experiments [19] showed that hormone binding by the ER was compromised upon Hsp90 inhibition. Geldanamycin functions by direct competition with ATP for binding to the N-terminal domain of Hsp90. Besides this direct effect, however, geldanamycin also de-represses the heat shock response leading to induction of other molecular chaperone proteins. Another effect of geldanamycin treatment is to stimulate degradation of steroid hormone receptors via the ubiquitin/proteasome pathway (see Ref. [20] for review). In view of these indirect effects of geldanamycin, we initiated a genetic approach to determine whether Hsp90 was required for hormone binding using yeast as a model system. We also analyzed whether Hsp90 is required for de novo folding of the ER in vitro using a mammalian cell-free system.

2. Materials and methods

2.1. Materials

17β-Estradiol and diethylstilbestrol (DES) were obtained from Sigma. The above compounds were solubilized in ethanol and stored at −20°C. Antisera to ER were a kind gift from Dr. G. Greene. Antisera to Hsp90 were prepared as described previously [21]. Antisera to yeast phosphoglycerate kinase was a kind gift from Dr. P. Lazarow. ^{3}H-DES and ^{3}H-17β-estradiol were purchased from American Radiochemical Company and NEN, respectively. Isogenic wild type and *G170D* mutant yeast strains were a kind gift from Dr. S. Lindquist. Plasmids encoding the *A97I* (pts38RV), *T101I* (pcs2-3RV) and *S485Y* (pts33BE) *hsp82* mutants were a kind gift from Dr. Y. Kimura and those encoding *E431K* (pTCA/hsp82 E431K), *G313N* (pTCA/hsp82 G313N) and *T525I* (pTCA/hsp82 T525I) were kind gift from Dr. K. Yamamoto.

2.2. Plasmid constructions

The wild type human ER open reading frame from p2HGPDER/CYC ([22]; gift from Dr. S. Lindquist) was subcloned into the vector pRS424 [23]. p2HGPDER/CYC was digested with SpeI and XhoI and the 3 kb insert containing the open reading frame of the HER was gel purified and subsequently subcloned into similarly digested pRS424 using standard methods. The resultant plasmid was designated pJR3.

2.3. Yeast methods and strains

Saccharomyces cerevisiae strains used in this study were derived from W3031a. Standard genetic methods were utilized for the growth and manipulation of the yeast *Saccharomyces cerevisiae*. These yeast strains were grown in either rich media (YPD) or selective media (SD) containing 0.67% yeast nitrogen base, 2% glucose with the additions of either adenine, uracil and/or the amino acids depending on auxotrophy. Yeast transformations were performed according to the method previously described [24].

The *hsp82* mutant strains (except for *G170D* which was a gift from Dr. S. Lindquist) were constructed from strain p82a [25] as previously described [26]. Yeast strains containing the *E431K* (pTCA/hsp82 E431K), *G313N* (pTCA/hsp82 G313N) or *T525I* (pTCA/hsp82 T525I) mutant alleles were transformed with the ER expression plasmid p2HGPDER/CYC [22]. Yeast strains containing *A97I* (pts38RV), *T101I* (pcs2-3RV) or *S485Y* (pts33BE) were transformed with the ER expression plasmid pJR3.

2.4. Ligand binding assays in yeast

Yeast cells were grown in selective media containing 2% glucose to early log phase ($OD^{600} = 0.2$) and 1 ml aliquots were subsequently incubated at either 25°C or 37°C for 30 min. Following this preincubation, cells were incubated with ^{3}H-DES for an additional 1.5 h at the same temperature. The cells were then washed three times with 1 ml of water each and counted in 5 ml of liquid scintillation fluid. Non-specific bound cpm was calculated by subtracting the cpm obtained from samples which were incubated with a 100 fold excess of unlabeled DES from the samples incubated in the absence of cold DES.

2.5. β-Galactosidase assay

Assays for β-galactosidase activity in yeast were performed as described previously using 17β-estradiol [26,27].

2.6. In vitro ligand binding assay

The ER gene was excised from p2HGPD/CYC using BamH1 and ligated into pBluescript vector. ER mRNA was transcribed using T7 polymerase in the

presence of $^{m7}G5'ppp5'G$ cap. This mRNA was added to rabbit reticulocyte lysates in the presence of 0.48% DMSO plus or minus various concentrations of geldanamycin. The reaction was then split into two and cold methionine (360 nM final concentration) was added to one half and ^{35}S-methionine (360 nM final concentration) added to the other to start the translation which was continued for 2 h at 30°C. At this time the translation performed in the presence of cold methionine was used for hormone binding experiments (see below) and the reaction performed in the presence of ^{35}S-methionine was denatured in SDS sample buffer, boiled and resolved in a denaturing polyacrylamide gel. Typically, 10% of the translation reaction was used for electrophoresis. The gel was fixed (10% acetic acid, 20% methanol), washed three times in water, incubated with 1 M sodium salicylate for 30 min then dried and exposed to X-ray film at −80°C.

Hormone-binding experiments were performed as follows. Reactions containing cold methionine (50 μl) were treated with ^{3}H-estradiol to a final concentration of 3 nM plus or minus a 400-fold excess of cold estradiol. The binding reaction was continued for 2 h at 4°C. At this time 100 μl hydroxylapatite slurry (50% w/v in 50 mM Tris–HCl pH 7.4, 1 mM EDTA) was added to the reaction and this was incubated on ice for 15 min with vortexing every 5 min. The reaction was diluted by adding 1 ml of ER wash buffer (40 mM Tris–HCl pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA), vortexed and the hydroxylapatite was pelleted at 13,000 × *g* for 2 min. The hydroxylapatite was washed twice more, resuspended in 200 μl of EtOH and added to 5 ml of scintillation fluid. The tube containing the hydroxylapatite was rinsed with a further 200 μl of EtOH and this was also added to the scintillation fluid. Specific binding was calculated by subtracting cpm from samples containing ^{3}H-labeled plus cold estradiol from samples containing only the ^{3}H-estradiol.

2.7. Western blot analysis

The levels of ER and Hsp90 were assayed by Western blot analysis using either anti-ER or anti-Hsp90 specific antibodies. Yeast lysates were prepared as previously described [27]. Lysates (10 μg total protein) were resolved by SDS-PAGE and the proteins in the gel were subsequently transferred to nitrocellulose (0.45 μ, MSI). Filters were briefly rinsed with TTBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) and blocked overnight at room temperature with TTBS containing 5% non-fat dry milk. Filters were incubated with antibodies for the ER or yeast Hsp90. Antibodies were diluted in antibody dilution buffer, 1 × PBS, 3% bovine serum albumin, 0.05% Tween 20 and 0.1% thimerosal (1:1000 for anti-Hsp90 and 1:2000 for anti-ER) for either 1 h (anti-Hsp90) or 4 h (anti-ER). Filters were washed three times for 10 min each in TTBS. Filters were then incubated with secondary antibody (HRP conjugated goat anti-rat IgG, diluted 1:2000 in antibody dilution buffer for anti-ER; and HRP conjugated goat anti-mouse IgG, diluted 1:10,000 in antibody dilution buffer for anti-Hsp90) for 1 h and subsequently washed three times for 10 min each in TTBS. Filters were treated with the chemiluminescence reagent (Pierce) and exposed to X-ray film. An identical filter was probed with anti-phosphoglycerate kinase (PGK; 1:300,000 dilution in antibody dilution buffer) to control for loading differences. The secondary antibody for the anti-PGK was HRP conjugated goat anti-rabbit IgG (1:10,000 in antibody dilution buffer). Washes and incubation times were identical to that for anti-Hsp90 and anti-ER.

3. Results

We initiated studies to test whether Hsp90 was required for hormone binding to the ER using methods that we previously described to assay hormone binding to the AR [11]. These studies were performed by incubation of ^{3}H-labeled diethylstilbestrol (DES), a synthetic estrogen, with live yeast cultures that were constitutively expressing the gene for wild type human ER. Ligand binding was assessed by quantitation of the amount of ligand retained by the cells after washing. We initially performed these studies with 17β-estradiol but found that there was a very high background of non-specific binding in yeast, as originally described by Burshell et al. [28] and Lyttle et al. [29]. This problem was overcome with the use of the synthetic estrogen DES for all our binding experiments as initially described by Lyttle [29].

3.1. DES Binding to the ER is defective in an hsp82 mutant yeast strains

To test whether Hsp90 is required for proper hormone binding by the ER, we expressed the receptor in isogenic wild type and *hsp82* mutant yeast strains. The mutant is isogenic to the wild type but contains a single point mutation, glycine to aspartate at position 170, in the *HSP82* gene. This mutation results in a temperature sensitive growth phenotype where the cells are viable at the permissive temperature of 25°C and inviable at restrictive temperatures above 33°C [25]. This mutant was originally characterized by Nathan and Lindquist to be defective in GR signaling [25]. In addition, previous studies with the *G170D* mutant showed that the AR binds R1881 (a synthetic androgen) like the wild type at 25°C but displays a reduced affinity at the restrictive temperature [11]. We therefore

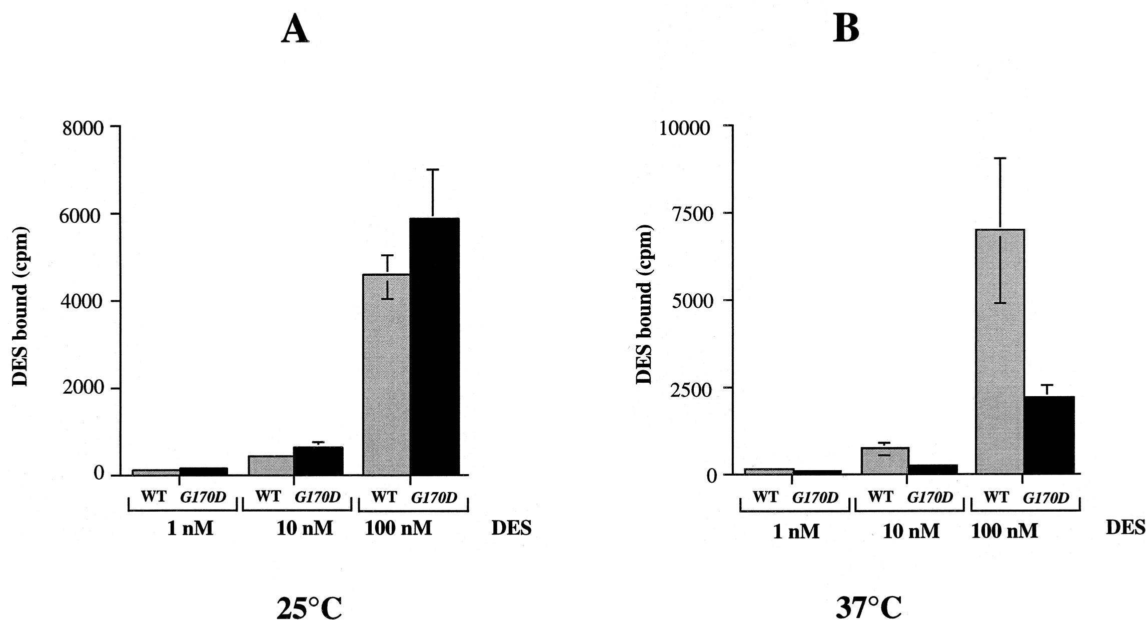


Fig. 1. Hormone binding to ER heterologously expressed in wild type and *hsp82*^{G170D} mutant yeast. Wild type (WT; gray bars) and *hsp82*^{G170D} (G170D; black bars) mutant yeast were incubated at 25°C (A) or 37°C (B) with 1, 10 and 100 nM (^{3}H) DES. Results are expressed as (^{3}H) DES bound (cpm). Results are the mean of three independent experiments.

expressed full length wild type ER in the wild type and *G170D* yeast strains and measured the binding of ^{3}H-DES to the receptors in live yeast cells. As shown in Fig. 1a, DES binds to a similar extent in both the wild type and *G170D* strains at concentrations ranging from 1 to 100 nM. The binding of DES in these cells was dependent on the presence of the ER, since in its absence the binding of DES was reduced to 12% background binding. At the restrictive temperature, however, there was a three-fold decrease in the capacity of ER in the *G170D* mutant cells to bind the DES, even though the wild type and mutant cells contained similar amounts of ER protein (see Fig. 4). These data indicate that the ER has a reduced capacity to bind DES upon Hsp90 loss of function, consistent with previous results using the AR expressed in these strains

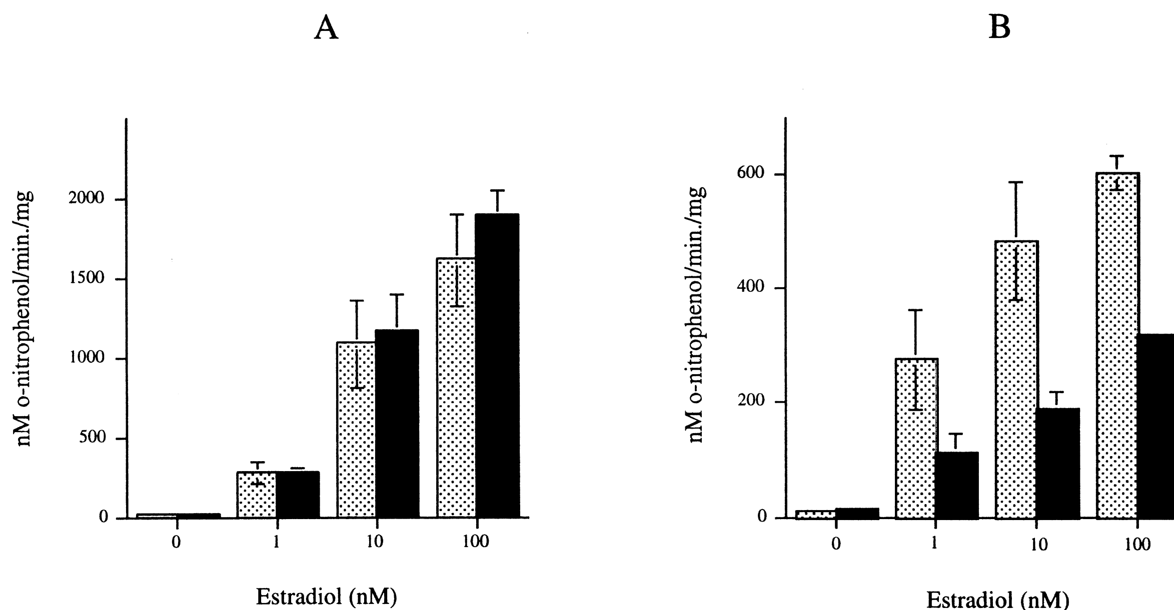


Fig. 2. β-Galactosidase activity assay in cell extracts from wild type (grey bars) and G170D mutant (black bars) yeast strains after treatment with 17β-estradiol at the concentrations shown. The assays were performed after incubation of hormone for 2 h with the strains at 25°C (A) or 37°C (B).

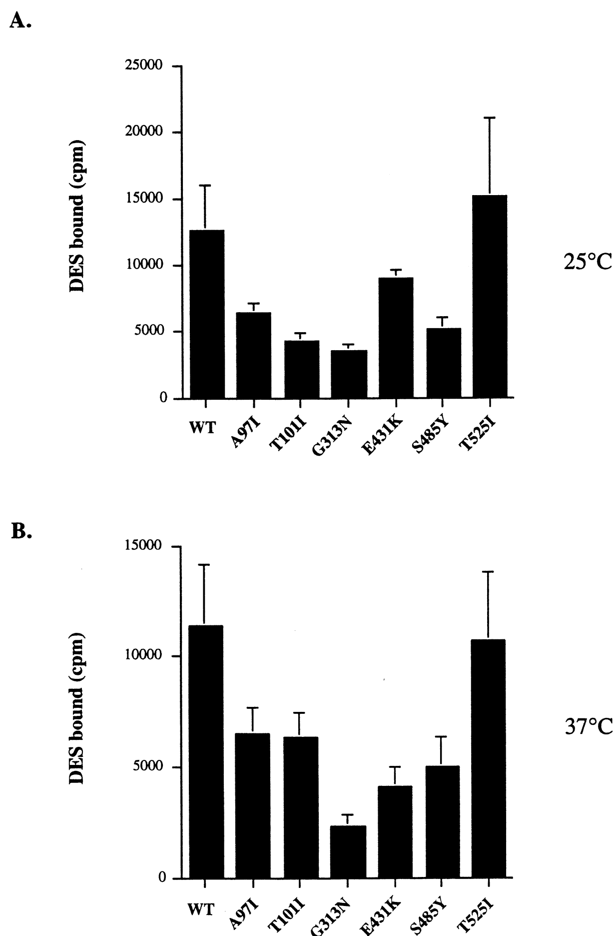


Fig. 3. Hormone binding by the ER in different *hsp82* mutant strains. (A) In vivo hormone binding experiments were performed with 100 nM DES at 25°C in the mutants listed in the figure. (B) Same as in (A) but the experiments were performed at 37°C. Each experiment was performed three times.

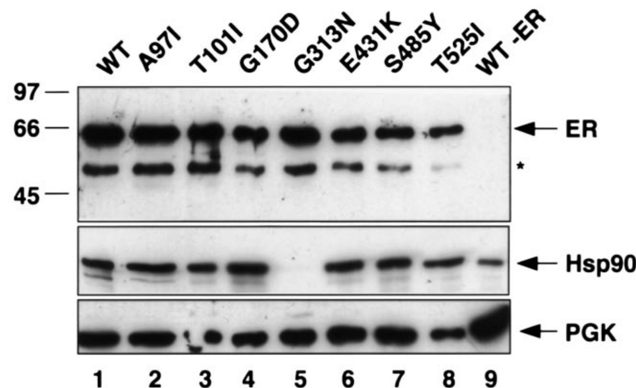


Fig. 4. Western blot analysis of ER (top panel; arrow denotes location of ER and star denotes breakdown product or ER), Hsp90 (middle panel; arrow denotes location of Hsp90). Analysis was performed on whole cell extracts of wild type expressing ER (WT; lane 1), *A97I* expressing ER (*A97I*; lane 2), *T101I* expressing ER (*T101I*; lane 3), *G170D* expressing ER (*G170D*; lane 4), *G313N* expressing ER (*G313N*; lane 5), *E431K* expressing ER (*E431K*; lane 6), *S485Y* expressing ER (*S485Y*; lane 7), *T525I* expressing ER (*T525I*; lane 8) and wild type not expressing ER (WT-ER; lane 9) yeast strains. Molecular weight standards are shown in kDa. Probing with antisera specific for Phosphoglycerate kinase was used as a loading control (PGK; lower panel; arrow denotes location of PGK).

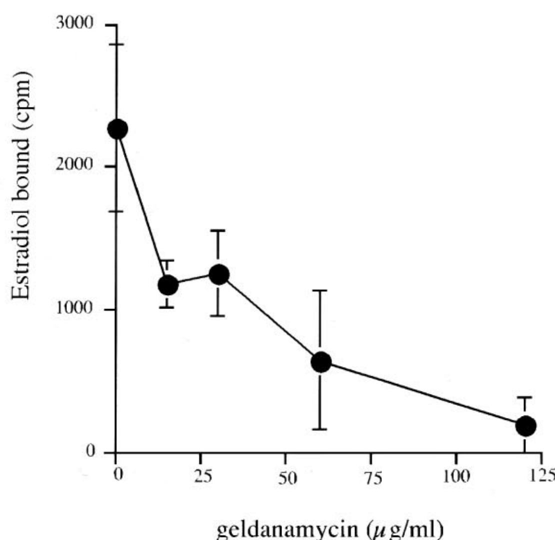
[11]. We also determined how loss of Hsp90 function affected the ability of the ER to transactivate a target *lac*Z gene in the presence of estradiol. As shown in Fig. 2, the ER stimulated *lac*Z gene expression to a similar extent in wild type and *G170D* mutant cells at 25°C but not at 37°C, where a two-fold decrease in activity was observed in the mutant.

Further analysis of hormone binding by the ER was performed with six different mutants of Hsp90. Three of these (G313N, E431K and T525I) were characterized previously as mutants that affected the function of GR expressed in yeast [30]. The other three (A97I, T101I and S485Y) were originally characterized as temperature sensitive growth mutants [31]. Hormone binding experiments were performed in each of these mutants after heterologous expression of wild type human ER. These experiments were performed by comparing the amount of hormone retained by the mutant cells with isogenic wild type cells after incubation with 100 nM DES. As shown in Fig. 3, there was a decrease in hormone binding in five out of the six mutants. The most severe defect was observed with the G313N mutant while the T525I mutant displayed hormone binding that was similar to the wild type at both 25 and 37°C.

Further characterization of these mutants was performed by Western blot analysis. As shown in Fig. 4, there were similar amounts of ER protein in each of the mutant strains compared to the wild-type. All of the strains also had a similar quantity of Hsp90 with the exception of the G313N mutant where it was barely detectable. Some Hsp90 protein was observed in this mutant upon overexposure of the Western blot (data not shown). The conclusion from these data is that Hsp90 is required for ER to bind hormone in a live cell environment. We next tested whether Hsp90 played a role in hormone binding by the ER in a mammalian cell-free system.

3.2. Inhibition of Hsp90 reduces hormone binding by the ER in vitro

Previous studies showed that geldanamycin inhibits Hsp90 by direct association with its N-terminal ATP-binding domain [32]. We therefore tested whether geldanamycin treatment of Hsp90 would affect the denovo folding of the ER in a cell-free system. For these experiments, ER mRNA was synthesized from a plasmid encoding the same wild type human ER gene used for the yeast studies. ER protein was synthesized from this mRNA in rabbit reticulocyte lysates (RRL) in the



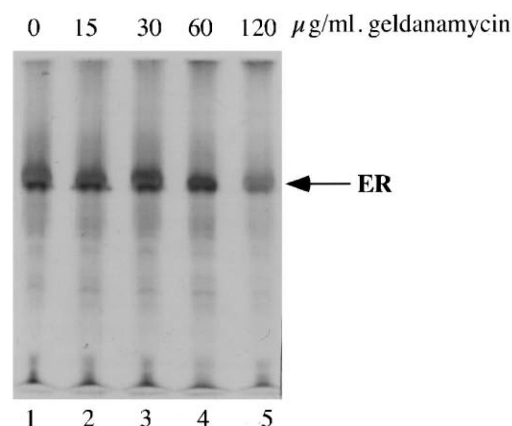


Fig. 5. Effect of geldanamycin on hormone binding by the ER expressed in a cell-free system. (A) ER mRNA was translated in rabbit reticulocyte lysates in the presence of different amounts of geldanamcyin. The reactions were stopped after 2 h and hormone binding determined as described in Materials and Methods. (B) ER protein levels after synthesis in presence of different amounts of geldanamycin. ER mRNA was synthesized in the presence of ^{35}S-methionine for 2 h and samples resolved in a polyacrylamide gel. The gel was dried and exposed to X-ray film.

absence or presence of various amounts of geldanamycin. We then assayed hormone binding with ^{3}H-β-estradiol by determining the amount of counts retained on hydroxylapaptite (see Section 2).

The results of these experiments, shown in Fig. 5, demonstrates a correlation between increased geldanamycin treatment and decreased hormone binding by the ER. As shown in Fig. 4B, however, geldanamycin also appears to affect the steady state amount of ER synthesized in RRL at higher concentrations although not at lower ($<$ 30 μg/ml) concentrations. These results indicate, therefore, that geldanamycin does inhibit hormone binding by the ER at low concentrations, since there was a two-fold decrease in hormone binding at 15 and 30 μg/ml geldanamycin without any decrease in the amount of ER protein compared with DMSO alone. Subsequent pulse chase studies indicated that geldanamycin inhibited ER synthesis in these lysates at concentrations over 30 μg/ml (data not shown).

4. Discussion

The results shown in this report demonstrate that human ER requires the molecular chaperone Hsp90 for efficient hormone binding. Two different assay procedures were used in these studies to determine the role Hsp90 in vivo (using the yeast system) and in vitro (in a mammalian cell-free system). In each case, there was reduced binding of ligands to the ER upon Hsp90 loss of function, via mutation or inhibition of action.

Studies from the yeast system revealed that different mutations in Hsp90 led to reductions in the level of hormone binding by the ER to varying extents The most dramatic decrease was observed with the G313N mutant, which also had the least amount of Hsp90 protein as determined by Western blot analysis (Fig. 4). The least affected was the T525I mutant which had almost wild type levels of hormone binding (Fig. 3). In a previous study by Bohen [30], the ER was unable to transactivate a target *lacZ* gene efficiently in this mutant, suggesting that the transactivation phenotype in T525I manifests at a stage downstream from hormone binding. A similar conclusion was drawn by Bohen concerning the E431K mutant. In this instance, transactivation of a target gene by GR was severely compromised in E431K, yet hormone binding was not impaired [33]. In the same mutant, we found that DES binding by the ER was substantially reduced. The reasons why this mutant affected hormone binding by ER but not by GR are not clear. The overall con-

clusion from these studies, however, is that Hsp90 is required for hormone binding by the ER in a live cell environment.

While our results are self-consistent with those from studies of other steroid receptors, they suggest that ER dynamics in vivo differ from those in vitro. Purified ER, for example, can bind hormone with high affinity in the absence of Hsp90 [13]. In an attempt to clarify this issue, we studied the role of Hsp90 on the de novo folding of the ER synthesized in vitro. The results of these studies were also consistent with a role for Hsp90, since low concentrations of geldanamycin reduced hormone binding without affecting ER protein levels. Our conclusion, therefore, is that Hsp90 assists in the de novo folding of the ER in vitro, although, once folded the ER is sufficiently stable to maintain a conformation that is competent for hormone binding. This situation may not occur in vivo where the environment is hot and crowded; conditions that are more conducive to protein aggregation than folding. In this case, Hsp90 may be required to assist in refolding of receptors that are constantly in equilibrium between folded and unfolded states. Support for this hypothesis is derived from the work of Nathan and Lindquist [25], who demonstrated that Hsp90 was constantly required for GR activation even after the initial folding event. Further studies suggest that steroid hormone receptors may exist in two pools, one inactive and the other active, at least in yeast. Conversion of the inactive pool to the active pool may depend on Hsp90 and its co-chaperones. In a recent study, it was found that overexpression of one Hsp90 co-chaperone called p23 was capable of increasing the activity of ER expressed in yeast [15]. This increase occurred without changes in the steady state levels of ER protein and correlated with increased levels of hormone binding.

In summary, our studies have shown that the ER requires Hsp90 for efficient hormone binding in yeast and in a cell-free system after de novo synthesis. This requirement probably reflects the role of Hsp90 and its co-chaperones in ER folding possibly at the level of the hormone-binding domain. These studies therefore demonstrate that Hsp90 has a similar role in ER activation as it does in the activation of other steroid hormone receptors such as AR, GR and PR.

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