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The Role of the Hsp90 Chaperone Machine in Androgen and Estrogen Receptor Action

by

Albert Edward Fliss Jr.

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1998

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THE CITY UNIVERSITY OF NEW YORK

Abstract

The Role of the Hsp90 Chaperone Machine in Androgen and Estrogen Receptor Action

by

Albert Edward Fliss Jr.

Adviser: Professor Avrom Caplan

Hsp90 along with its binding proteins or co-chaperones comprises the Hsp90 chaperone machine. This chaperone machine is important in the regulation of steroid hormone receptor activation and is functionally conserved from yeast to mammals. In the present study, the yeast model system was utilized to determine the role of Hsp90 and two of its co-chaperones Ydj1 and Cdc37 in AR and ER hormone binding and subsequent activation. Results from direct hormone binding and ligand competition assays suggest that both Hsp90 and Ydj1 are required for hormone binding to both the AR and ER. In the absence of functional Hsp90, AR and ER were decreased in their ability to bind hormone. Likewise, the ability of hormone antagonist to compete for hormone binding to either receptor was altered. In the absence of Ydj1 or functional Hsp90, the AR antagonist HF was converted from a weak competitor of R1881

binding to a potent one. In contrast, the ER antagonist 4-OHT was converted from a competitor to a potentiator of DES and E2 binding to the ER in the absence of functional Hsp90, whereas, it neither competes nor potentiates DES binding in the absence of Ydj1. The defect in AR hormone binding seen in the Δydj1 strain is mediated via the J domain of Ydj1 and Hdj2 was able to fully complement the AR signaling defect.

Previous studies have demonstrated that Cdc37 is also part of the Hsp90 chaperone machine. In this study, it was demonstrated that hormone dependent AR signaling was grossly defective in a Cdc37 mutant strain and this defect was mediated via the HBD. However, in contrast to Hsp90 and Ydj1, the Cdc37 defect was not in hormone binding. This defect was seen to a lesser extent with GR and ER, suggesting that Cdc37 differentially regulates steroid hormone receptor activation.

In summary, Hsp90 and Ydj1 are required for hormone binding to the AR and ER. Cdc37 is differentially required for hormone dependent activation, however, this defect is not in hormone binding. This demonstrates that components of the chaperone machine can act before or after hormone binding.

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I would also like to thank my parents for giving me strength, encouragement and love I have needed to complete my education and to always strive for more. I would like to express my deepest appreciation and love to my wife, Makiko, for her never-ending support , critical advice and love and for putting up with me during the preparation of my thesis. Makiko and Snowey always provide me will a wonderful enviroment to live in.

I would like to dedicate this thesis to my son, Nicholas. For his birth is truely one of the greatest things that has happened in my life. I thank God for him everyday of my life.

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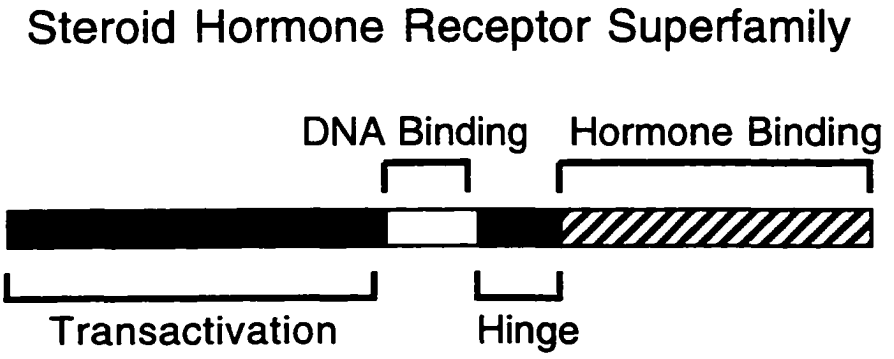
CHAPTER 1

BACKGROUND AND INTRODUCTION

INTRODUCTION

The androgen (**AR**) and estrogen (**ER**) as well as the glucocorticoid (**GR**), progesterone (**PR**) and mineralocorticoid (**MR**) receptors are members of the steroid hormone receptor superfamily (For review, Yamamoto, 1985; Evans, 1988). All of these molecules are intracellular nuclear receptors that become active transcription factors upon hormone binding. All members of the steroid hormone receptor superfamily have structural similarities, each contains a non-conserved amino-terminal transactivation domain which is responsible for interacting with the basal transcriptional machinery in order to modulate target gene expression. There is also a highly conserved DNA binding domain which is responsible for interacting with hormone response elements in the promoter region of target genes. Next to the DNA binding domain is a flexible hinge region. And finally on the carboxy-terminus of the receptor, there is the conserved hormone binding domain **(HBD)** which is the portion of the receptor that binds hormone (Figure 1).

Hormone binding triggers a number of events including the induction of conformational changes which are transmitted to the DNA binding and transactivation domains. The hormone-bound receptor is able to interact with its cognate HRE (hormone response element) and transactivate it's target genes. So perhaps the first crucial step in the activation of steroid hormone



Hormone Dependent Activation

1. Hormone binding
2. Transmission of conformational changes
3. Transactivation and DNA binding

Figure 1. **Steroid Hormone Receptor Structural Organization and Function**

receptors is the binding of hormone at high affinity. Dissociation constants (K_d's) of the high affinity binding sites of most steroid receptors are typically in the low nanomolar range. Affinity labeling and mutagenesis studies have demonstrated that hormone-receptor interactions occur via the HBD of the ER and the AR (Katzenellenbogen et al, 1993; Wong et al, 1995). Recent crystalization studies have demonstrated that the hormone binding pockets of the thyroid hormone (**THR**) (Wagner et. al., 1995), retinoic acid (Renaud et. al., 1995), ER (Brzozowski et. al., 1997; Tanenbaum et. al., 1998) and PR (Tanenbaum et. al., 1998) are hydrophobic in nature. So in order to attain high affinity binding, the lipophilic hormone must gain access to the hydrophobic binding pocket of the receptor. In order to accomplish this task, conformational alterations or refolding must occur in the HBD. Since it is possible that the hydrophobic pocket might collapse in the absence of hormone. These alterations may in part be facilitated by molecular chaperones.

Molecular chaperones play important roles in both protein folding and signal transduction. Molecular chaperones were defined by Hartl as "proteins that bind to and stabilize an otherwise unstable conformer of another protein, and by controlled binding and release of the substrate protein facilitates its correct fate *in vivo* , be it folding, oligomeric assembly, transport to another subcellular compartment or controlled switching between active and inactive conformations" (Hendrick and Hartl 1993). Of the molecular chaperones only the Hsp90 chaperone machine plays a role in signal transduction (Figure 2).

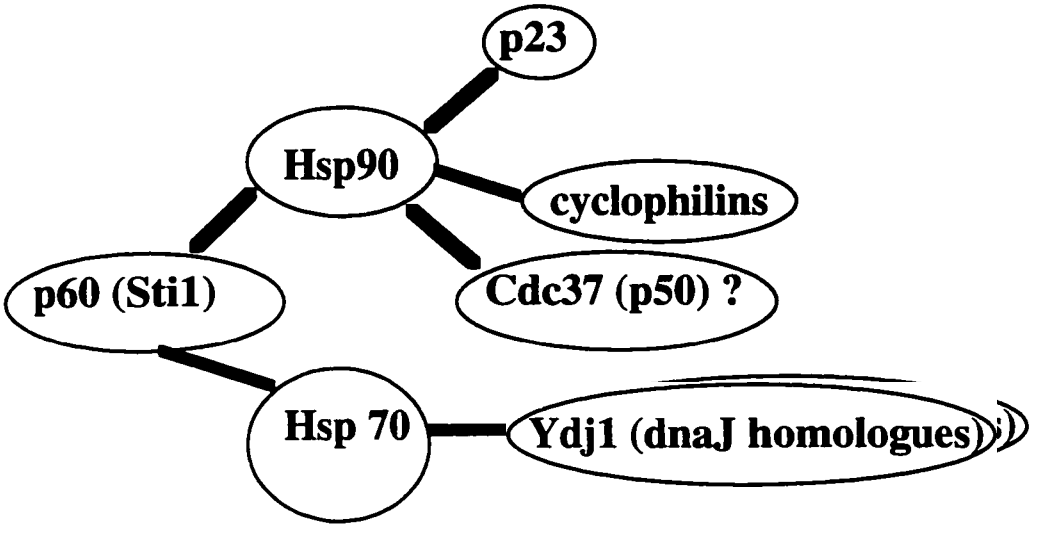


Figure 2. **The Hsp90 Chaperone Machine**

[1] The Hsp90 Chaperone Machine

The Hsp90 chaperone machine is comprised of Hsp90 and its many binding proteins or co-chaperones. Hsp90 is found in the unliganded complexes of the AR (Mariovet et al, 1992; Sullivan et. al., 1985), ER (Redeuilh et al, 1987), PR (Sullivan et. al., 1985; Nemoto et al, 1992; Schuh et al, 1985; Radanyi et al, 1989), GR (Sanchez et al, 1985; Sullivan et. al., 1985), and MR (Rafestin-Oblin et al, 1989). Two molecules of Hsp90 are bound to each aporeceptor complex (Radanyi et al, 1989). In addition to Hsp90, there are several other co-chaperone molecules contained in these aporeceptor complexes, including Hsp70, p60 (Sti1), p48 (Hip), p23 and cyclo/immunophilins (Nair and Smith, 1996; Smith et , 1990 a and b; Smith and Toft, 1992; Johnson and Toft, 1994). These components of the Hsp90 chaperone machine exist in two distinct subcomplexes (Smith, 1993). The first of these complexes is comprised of Hsp90, Hsp70 and p60/Sti1 and is sufficient for hormone binding to the GR *in vitro* (Dittmar and Pratt, 1997). The second of these subcomplexes consists of Hsp90, p23/SBA1 and one of the many cyclo/immunophilins and is thought to stabilize the hormone bound receptor complex (Figure 3) (Johnson et al., 1994; Smith et. al., 1990a; Hutchison et. al., 1994; Hutchison et. al., 1995; Johnson and Toft, 1995). Upon hormone binding, the aporeceptor complex is converted to an active receptor that is capable of binding DNA and transactivating target genes (For review, Evans, 1988). This shift from inactive to active receptor is accompanied by the dissociation of Hsp90 (Kost et al, 1989)

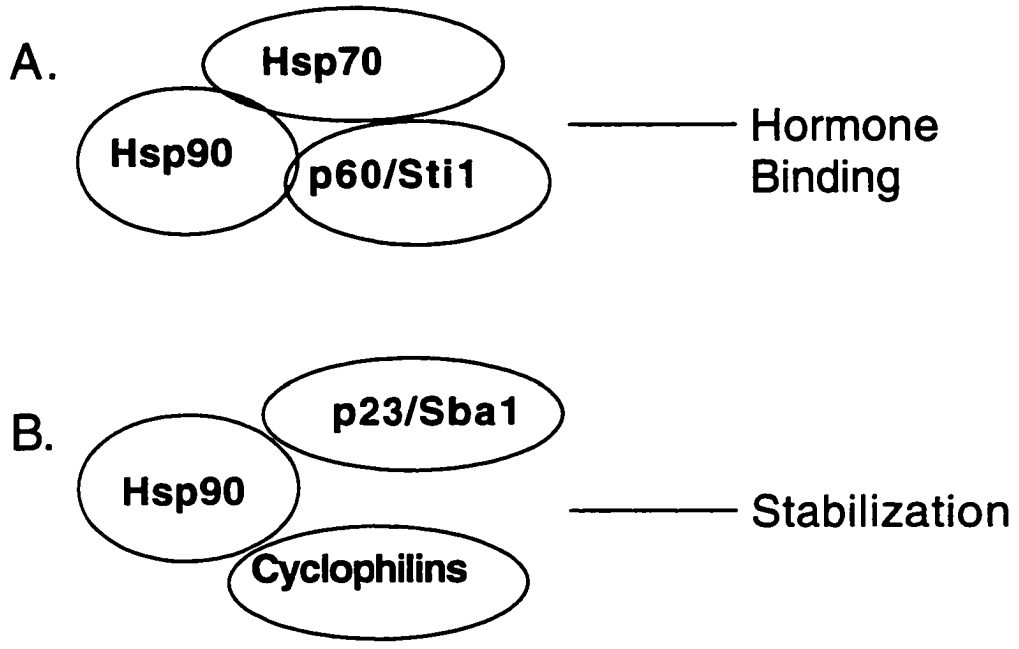


Figure 3. **Hsp90 Subcomplexes**

[2] Hsp90

Yeast have been extensively used as a model system to study the role of Hsp90 in steroid hormone receptor activation. Hsp90 is an essential for growth in the yeast and is coded for by the*HSP82* and the *HSC82* genes (Borkovich et. al., 1989). Of these two genes, *HSC82* is constitutively expressed at high levels, whereas, *HSP82* is heat shock inducible (Finkelstein and Strausberg, 1983; Borkovich et. al., 1989). The Hsp90 chaperone machine is functionally conserved from yeast to mammals (Chang and Lindquist, 1994) (Table 1). Mammalian target proteins are able to physically and functionally interact with yeast homologues of mammalian molecular chaperones. Likewise, steroid receptors can function in a hormone dependent manner when introduced into yeast (Picard et al, 1990). Results from *in vivo* studies with yeast strains that are either deficient in Hsp90 or contain temperature sensitive *hsp82* mutants suggest that Hsp90 is required for ER (Picard et al, 1990), GR (Picard et al, 1990; Bohen and Yamamoto, 1993; Bohen, 1995; Nathan and Lindquist, 1995), MR (Picard et al, 1990), PR (Bohen and Yamamoto, 1993), and retinoic acid receptor (**RAR**) (Holley and Yamamoto et al, 1995) activation. Likewise, previous studies have suggested that this decrease in transactivation is at least in part due to a defect in hormone binding. Both *in vitro* and *in vivo* experiments have demonstrated that Hsp90 is required for high affinity hormone binding to the GR (Bresnick et al, 1989; Picard et al, 1990; Bohen and Yamamoto, 1993; Bohen, 1995). Since GR was unable to bind hormone at 4°C in the absence or depletion of

TABLE 1: The Hsp90 Chaperone Machine

Component	Mammalian Cell	Yeast Cell Counterpart	Yeast Gene
Hsp90	yes	yes	HSC82/HSP82
Hsp70	yes	yes	SSA1/SSA2/SSA3/SSA4
p60/Hop	yes	yes	STI1
p50	yes	yes	CDC37
p48/Hip	yes	no	---
Cyp-40	yes	yes	CPR 6/CPR 7
dnaJ	yes	yes	YDJ1
p23	yes	yes	Sba1

functional Hsp90 (Bresnick et al, 1989). Likewise, GR was unable to bind hormone when introduced into yeast that contained a temperature sensitive *hsp82* mutant. Similarly, MR also requires Hsp90 for high affinity hormone binding (Schulman et al, 1992).

In contrast to GR and MR, previous studies have suggested that Hsp90 is not needed for high affinity hormone binding to the AR and ER (Binart et al, 1995; Nemoto et al, 1992), implying that Hsp90 is not required for hormone binding to all steroid receptors. The caveat being these experiments were performed at non-physiological temperature under *in vitro* conditions in which molecular chaperone may not normally be required. However, since molecular chaperones themselves are needed to block off-pathway inter and intra-molecular interactions under conditions of high protein concentration and high temperature, this may put into question the physiological relevance of these *in vitro* experiments. In contrast, Segnitz and Gehring recently reported that Hsp90 is required for hormone binding to the ER *in vivo* (Segnitz and Gehring, 1997). In these experiments, ER had a decreased ability to bind hormone when mammalian cells were treated with the Hsp90 inhibitor, geldanamycin (anti-tumor agent), suggesting that Hsp90 is also required for hormone binding to the ER (Segnitz and Gehring, 1997). PR also has a conditional requirement for Hsp90 in hormone binding. PR was unable to bind hormone in the absence of Hsp90 (Smith, 1993) *in vitro* at 37°C, whereas, it's binding was recovered at 4°C. This suggests that Hsp90 requirements may be different for each receptor, possibly revealing an inherent difference in

receptor structure. Taken together, these studies strongly suggest that Hsp90 may be essential for high affinity hormone binding to most steroid hormone receptors.

[3] The Role Of Co-Chaperones In Steroid Hormone Receptor Activation

[A] Hsp70

Along with Hsp90 in the chaperone machine exists several co-chaperones or binding proteins. Of these, Hsp70 is one of the co-chaperones that plays an important role in steroid hormone receptor activation. Hsp70 is a well characterized chaperone that is involved in protein folding (For review see Hartl et. al., 1992). During the process of protein folding, Hsp70 molecular chaperones bind and release polypeptides in a reaction cycle that is dependent upon ATP hydrolysis (Gething and Sambrook, 1992). Hsp70 itself exhibits a low level of ATPase activity, but this is augmented by dnaJ (Liberek et al, 1991a) and other J proteins including Ydj1 (Tsai and Douglas, 1996) (the yeast homologue of E. coli dnaJ). Hsp70 in its ATP bound state has a high on and high off rate for interactions with polypeptide substrates, whereas, in its ADP bound state, Hsp70 has a high on and low off rate for interactions with polypeptide substrates (Liberek et al, 1991b). Thus, Hsp70-ADP is able to stably interact with polypeptide substrates.

Hsp70 is present in unliganded steroid hormone receptor complexes (Diehl and Schmidt, 1993; Srinivasan et al, 1994; Ratajczak et al, 1993; Smith et al, 1990b), and is required for the stable association of Hsp90 with steroid hormone receptors in an ATP dependent manner (Hutchison et al, 1994; Smith et. al., 1992). When Hsp70 is depleted *in vitro*, Hsp90-GR complexes are not formed, nor are the receptors converted to their high affinity hormone binding state (Hutchison et al, 1994), suggesting that Hsp70 is required for high affinity hormone binding. Likewise, depletion of Hsp70 with antibody inhibits Hsp90 binding to PR (Smith et. al., 1992).

[B] Ydj1

As mentioned earlier, one of the co-chaperones that plays a crucial role in the Hsp70 reaction cycle is Ydj1p (the yeast homologue of E. coli dnaJ) (Caplan et. al., 1991). Ydj1p functions as a molecular chaperone and is able to prevent protein aggregation via a cysteine-rich zinc finger and carboxy-terminal domain (Lu and Cyr, 1998). All J proteins also contain a characteristic amino-terminal J domain (For review see Cheetham and Caplan, 1998). This characteristic J domain along with the glycine and phenylalanine rich region is responsible for J proteins ability to stimulate Hsp70's ATPase activity (Wall et al, 1994). Using the yeast model system, Caplan et al (1995) demonstrated that Ydj1 is required for the *in vivo* activation of AR. Hormone-dependent activation of the AR was decreased in yeast strains containing

either a total deletion or mutation (*ydj1-151*- contains multiple point mutations in all domains except the J domain) of YDJ1. This decrease in hormone dependent transactivation seen in the *ydj1* mutant yeast was mediated via the HBD (Caplan et al, 1995). A similar study demonstrated that a yeast strain containing a *ydj1*^{G315D} (contains a mutation in carboxy-terminal domain of Ydj1p) mutant allele caused derepression of hormone induced ER and GR activation. In these experiments, yeast strains containing the *ydj1*^{G315D} mutant allele had increased levels of hormone independent activity (Kimura et al, 1995). Likewise, *in vitro* studies using a reconstitution system also support the role of Ydj1 in hormone binding to the GR (Dittmar et. al, 1998). In this study, hormone binding to reconstituted GR:Hsp90:p60:Hsp70 was decreased in the absence of Hsp40 and this defect was reversed upon the addition of Ydj1p. Taken together these previous studies suggest a possible dual role for Ydj1 in steroid receptor activation.

[C] p60/Sti1

The process of ADP/ATP nucleotide exchange and subsequent polypeptide release is crucial for the recycling of Hsp70 and is thought to be stimulated by p60(Hop) (Gross et al, 1996a; Gross et al, 1996b). p60 is able to bind to both Hsp70 and Hsp90 and is also present in the aporeceptor complex (Chen et. al., 1996). Sti1 is the yeast homologue of mammalian p60(Hop) (Nicolet and Craig, 1989). In *Δsti1* yeast, GR are defective in their ability to be activated

upon hormone induction (Chang et al, 1996), while there is a simultaneous increase in the amount of Ydj1 interacting with the GR. These results suggest that Ydj1 plays a role at an earlier stage in the activation pathway of steroid receptors than does Sti1.

[D] p48/HIP and Bag1

In addition to DnaJ homologues and p60/Sti1, the Hsp70 reaction cycle is aided by additional co-chaperones which are also found in association with steroid hormone receptor complexes. These co-chaperones include p48/HIP (Hsp70 Interacting Protein) (Hohfeld et. al., 1995) and Bag1 (Yakayama et. al., 1996). p48/HIP is thought to stabilize the ADP bound state of Hsp70 and may have additional chaperoning functions of its own (Hohfeld et. al., 1995). Recently, Prapapanich et al demonstrated that mutation of the carboxy-terminal region of HIP causes a dose dependent defect in the ability of PR to assemble in a complex with Hsp90, suggesting that HIP is important in receptor signaling (Prapapanich et. al., 1998). Bag1 was initially isolated as an anti-apoptotic protein which was able to bind Bcl-2 (Yakayama et. al., 1996). A Bag1 homologue was later demonstrated in association with activated GR and ER (Zeiner and Ulrich, 1995) and is thought to have GrpE-like activity (Hohfeld and Jentsch, 1997). To date little is known about the specific role of either

p48/HIP or Bag1 in steroid hormone receptor activation other than their association with the Hsp70 reaction cycle.

[E] p23/Sba1

The 23-kDa acidic protein, p23/Sba1, is also present in the aporeceptor complexes of the ER (Ratajczak et al, 1993), PR (Smith and Toft, 1990; Smith et al, 1990a; Smith et. al., 1990b; Johnson et. al., 1994; Johnson and Toft, 1994) , and GR (Hutchison et. al., 1995; Dittmar et al, 1996). Results from experiments performed in vitro suggest that p23 is required for hormone binding to the GR and PR (Dittmar et. al., 1997; Hutchison et al, 1995; Johnson and Toft, 1994; Smith et al, 1995; Johnson and Toft, 1991; Hutchison et. al., 1994). Upon depletion of p23, GR are unable to bind hormone at 4°C (Hutchison et al, 1995). Similarly, when the interactions between Hsp90 and p23 are disrupted, hormone binding to PR is abolished (Smith et al, 1995) and there is a concurrent loss of the cyclophilin, CyP40, from the receptor (Johnson et al, 1995). However, *in vivo* yeast studies have failed to reveal any role for p23 in steroid receptor activation (Fang et al., 1998, Bohen 1998). In these studies, both AR and GR are able to be fully activated by hormone in the absence of p23, suggesting that p23 may not be required *in vivo*.

[F] Cyclophilins

Cyclophilins similar to CyP40 are found in both ER, PR and GR complexes (Hutchison et al, 1993; Smith et. al., 1993a; Smith et. al., 1993b; Johnson and

Toft, 1994). Recent *in vivo* studies have demonstrated that yeast lacking Cpr7, the yeast homologue of CyP-40, show a decrease in hormone dependent GR activation (Duina et al, 1996). Picard (1996) has recently demonstrated that there is no GR transactivation defect when comparing a Δcpr6 mutant with its isogenic wild type strain (Warth et. al., 1997). This suggests that only Cpr7 plays an important role in receptor signaling *in vivo* .

[G] p50/Cdc37

Although its presence in steroid hormone aporeceptor complexes has not been demonstrated (Nair et al, 1997, Kimura et al., 1997), Cdc37 can physically interact with Hsp90 in the activation of certain kinases including v-src kinase (Brugge, 1986; Dey et al, 1996), CDK4 (Stepanova et al, Dai et al., 1996) and the Sevenless kinase of Drosophila (Cutforth and Rubin, 1994). Likewise, Hsp90, Ydj1, Sti1, and Cdc37 are all suppressors of the v-src lethality phenotype in yeast (Kimura et. al., 1995; Nathan and Lindquist, 1995; Dey et. al., 1996a; Dey et. al., 1996b; Chang et. al., 1997). In wild type yeast, the expression of v-src leads to a lethal phenotype due to the inappropriate phosphorylation of many proteins at their tyrosine residues (Florio et. al., 1994). However in yeast that contain a mutation in any of these genes, v-src has decreased kinase activity and the lethal phenotype is suppressed (Xu and Lindquist, 1993; Kimura et. al., 1995; Nathan and Lindquist, 1995; Dey et. al., 1996a; Dey et. al., 1996b; Chang et. al., 1997). This might suggest a role for

p50/Cdc37 in other signaling pathways involving the Hsp90 chaperone machine.

Taken together these previous studies demonstrate that the individual components of the Hsp90 chaperone machine play vital roles in steroid hormone receptor activation. I will now describe in my thesis what role Hsp90, Ydj1 and Cdc37 play in hormone binding and activation of the AR and ER *in vivo*.

CHAPTER II

CHARACTERIZATION OF THE ROLE OF HSP90 IN ANDROGEN AND ESTROGEN RECEPTOR ACTION

Introduction

Hsp90 plays an important role in the regulation of signal transduction by steroid hormone receptors and protein kinases. In the case of steroid hormone receptors, Hsp90 itself associates with the unliganded forms of the AR (Mariovet et al, 1992; Sullivan et. al., 1985), ER (Redeuilh et al, 1987), GR (Sanchez et al, 1985; Sullivan et. al., 1985), MR (Rafestin-Oblin et al, 1989) and the PR (Nemoto et al, 1992; Schuh et al, 1985; Radanyi et al, 1989; Sullivan et. al., 1985). Although Hsp90 binds to the unliganded forms of all the above mentioned receptors, there is little consensus for Hsp90 function. For example, it has been found from experiments performed *in vitro* that Hsp90 is essential for hormone binding to the GR under all conditions (Bresnick et. al., 1989) , whereas, it is required for high affinity hormone binding to the PR only at 37°C (Smith, 1993). Likewise, Hsp90 appears to be important for the ability of ER to bind hormone with high affinity *in vivo* in animal cells (Segnitz and Gehring, 1997). In this study, treatment of animal cells with geldanamycin (a benzoquinoid ansamysin that directly binds to Hsp90 and inhibits its function) resulted in reduced levels of hormone binding to several receptors including ER (Segnitz and Gehring, 1997). However, previous *in vitro* studies suggest that Hsp90 is not required for hormone binding to the AR or ER (Nemoto et. al., 1992; Binart et. al., 1995). In these studies both the AR and ER were able to bind hormone in the absence of Hsp90. However as explained earlier, these experiments were performed in vitro at 4°C with a truncated recombinant

receptor construct. Thus, it appears likely that molecular chaperones modulate the activity of steroid hormone receptors *in vivo*, although they may not be required under certain experimental conditions *in vitro*.

Analysis of the Hsp90 mechanism of action has been largely restricted to studies with the GR and PR. It has been demonstrated that Hsp90 functions via discrete sub-complexes that may bind to the receptors independently of each other. The first Hsp90 sub-complex containing Hsp90/Hsp70/p60 (Sti1p) and p48 (Hip) appears to be important for generating the high affinity hormone binding conformation (Dittmar and Pratt, 1997). Once formed, this conformation is stabilized by another complex that includes Hsp90, one of several cyclo/immunophilins and p23/Sba1 (Johnson et. al., 1994; Smith et. al., 1990a; Hutchison et. al., 1994; Hutchison et. al., 1995; Johnson and Toft, 1991). It has been proposed that receptors continually cycle in and out of the high affinity hormone binding conformation, and in yeast, Nathan and Lindquist showed that Hsp90 was continuously required for hormone dependent activation of GR (Nathan and Lindquist, 1995) .

In the study presented here, the yeast system was used in order to determine the role of Hsp90 in ligand binding to the AR and ER. One may ask why use yeast to study mammalian steroid receptors that are normally not present in yeast? First, the Hsp90 chaperone machine is functionally conserved from yeast to mammals allowing one to determine its role in steroid receptor action in an *in vivo* setting. Second, the genetic malleability of *Saccharomyces cerevisiea* allows one to dissect the role of the individual

components of the chaperone machine in steroid receptor activation. Finally, previous studies have demonstrated that steroid receptor including the AR and ER are able to function in a hormone dependent manner when heterologously introduced into *Saccharomyces cerevisiea.* Our results using the yeast system indicate that Hsp90 operates at a distinct step in generating the high affinity hormone binding state of the AR and ER. Using both hormone binding and ligand competition assays, it was demonstrated that AR and ER are decreased in their ability to bind hormone in the absence of functional Hsp90. In addition, hydroxyflutamide **(HF)** was converted from a weak to a strong competitor of hormone binding to the AR in the absence of functional Hsp90. Interestingly, 4-hydroxytamoxifen **(4-OHT)** was converted from a strong competitor to a strong potentiator of hormone binding to the ER in the absence of functional Hsp90. This suggests that Hsp90 is required for ligand binding to both the AR and the ER.

Results

Previous *in vivo* studies in the yeast system revealed that Hsp90 was required for activation and high affinity hormone binding to the GR (Bohen and Yamamoto, 1993; Bohen, 1995). Studies were therefore initiated to test whether Hsp90 was also required for high affinity hormone binding to the AR and ER. These studies were performed by incubating ^{3}H labeled hormone with wild type or Hsp90 mutant yeast transformed by a plasmid constitutively

expressing either the AR or the ER. Hormone binding was assessed by quantitating of the amount of labeled ligand retained by the cells.

[1] DES Binding to the ER is Defective in an *hsp82* Mutant Strain

To test whether Hsp90 is required for proper hormone binding to the ER, the full length human receptor was constitutively expressed in both the isogenic wild type and an *hsp82* mutant yeast strain. The mutant used in the hormone binding studies contained a single point substitution, glycine to aspartate at position 170, in the *HSP82* gene, and is termed *G170D*. This mutation results in a temperature sensitive growth phenotype where the yeast are viable at the permissive temperature of 25°C and inviable at restrictive temperatures above 33°C (Kimura and Yahara, 1994; Nathan and Lindquist, 1995). This mutant was originally characterized by Nathan and Lindquist to be defective in GR signaling (Nathan and Lindquist, 1995). In order to construct both the wild type and *hsp82* mutant strains used in this study, both the HSC82 and HSP82 alleles were disrupted and replaced by a plasmid which constitutively expresses either the wild type or mutant allele (Nathan and Lindquist, 1995). Direct hormone binding assays were performed by incubating growing yeast which were heterologously expressing ER with ^{3}H diethylstilbesterol **(DES).** As shown in Figure 4A, DES binds to a similar extent in both the wild type and *G170D* strains at concentrations ranging from 1-100 nM at the permissive temperature of 25°C and this binding was dependent on the presence of the ER. At the restrictive temperature, however,

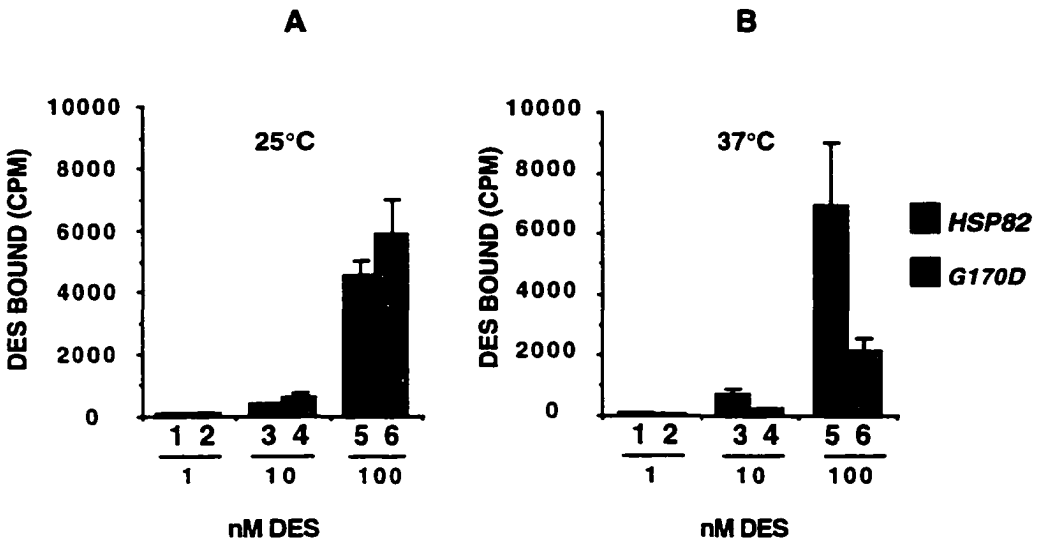


Figure 4. **Hormone binding to ER heterologously expressed in wild type and *hsp82*^{G170D} mutant yeast.** Wild type strain AFY43 (*HSP82*; black bars) and *hsp82*^{G170D} (*G170D*; gray bars) mutant yeast strain AFY44 heterologously expressing ER were incubated at 25°C (A) or 37°C (B) with 1 (lanes 1 and 2), 10 (lanes 3 and 4) and 100 (lanes 5 and 6) nM [^{3}H] DES. Results are expressed as [^{3}H] DES bound (cpm). Results are the mean of at least three independent experiments.

there was a 3-fold decrease in the ability of ER to bind the DES (100 nM) in the *G170D* mutant cells (Figure 4B-lane 5 and 6), even though the wild type and *hsp82* mutant cells contained similar amounts of ER protein (see later, Fig.8). These data indicate that the ER is decreased in its ability to bind DES upon Hsp90 loss of function.

[2] 4-OHT Increases DES Binding to the ER Upon Hsp90 Loss of Function

Previous studies have demonstrated that both hormone and hormone antagonists interact with similar residues in the hydrophobic ligand binding pocket of steroid hormone receptors (Katzenellenbogen et al, 1993; Wong et al, 1995; Brzozowski et. al., 1997), suggesting that there may be a concurrent alteration in the ability of a hormone antagonist to compete for hormone binding. To see if this was the case for the ER, it was tested whether decreased binding of DES to the ER in the *G170D* mutant would manifest itself in any alteration in the ability of the drug 4-OHT to compete for DES binding.

When wild type yeast cells containing the ER were incubated with DES in the presence of increasing concentrations of the competitive inhibitor 4-OHT, there was an expected dose dependent decrease in the level of DES binding, at both 25°C and 37°C (Figure 5A and B-lanes 3, 5, and 7). However, this decrease was not observed when these same experiments were performed with the ER in the *G170D* mutant strain (Figure 5A and B-lanes 4, 6, and 8). In the *G170D* mutant, 4-OHT was unable to compete for DES binding in *G170D*

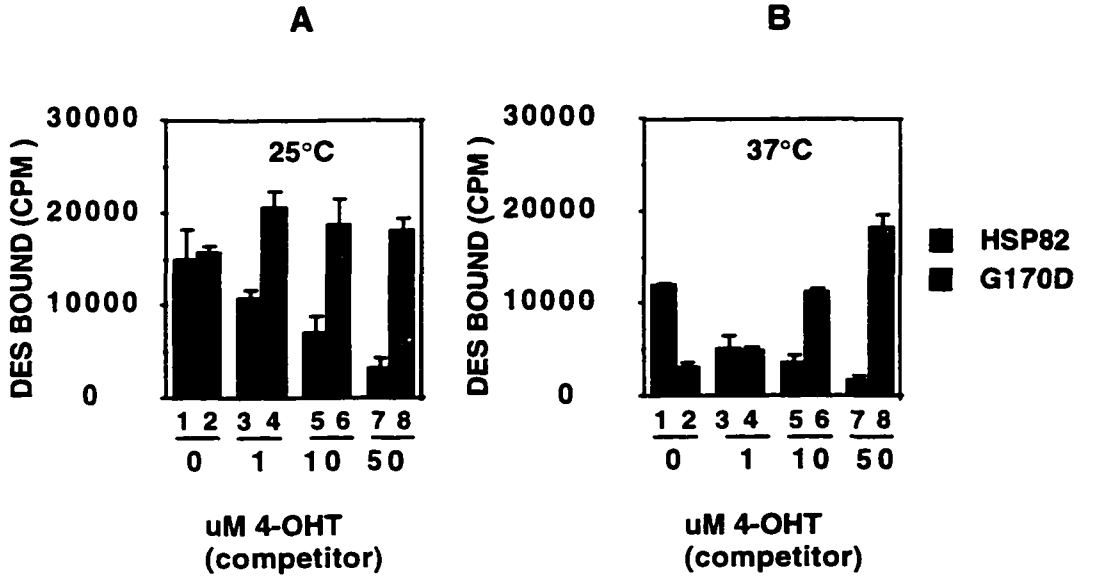


Figure 5. **4-OHT competition assay with DES in wild type and *hsp82*^{G170D} mutant yeast.** (A) Wild type strain AFY43 (*HSP82*; black bars; lanes 1,3,5,7) and *hsp82*^{G170D} (*G170D*; gray bars; lanes 2,4,6,8) mutant yeast strain AFY44 heterologously expressing ER were incubated at 25°C with or without varying amounts (1 (lane 3), 10 (lane 5) and 50 (lane 7) μM) of 4-OHT in the presence of 100 nM [^{3}H] DES. (B) Wild type (WT; gray bars; lanes 1,3,5,7) and *hsp82*^{G170D} (*G170D*; black bars; lanes 2,4,6,8) mutant yeast heterologously expressing ER were incubated at 37°C with or without varying amounts (1 (lane 3), 10 (lane 5) and 50 (lane 7) μM) of 4-OHT in the presence of 100 nM [^{3}H] DES. Results are expressed as [^{3}H] DES bound (cpm x 10^{-2}). Results are the mean of at least three independent experiments.

at 25°C (Figure 5A-lanes 4, 6, and 8) , whereas, 4-OHT actually increased DES binding (7.5 fold increase) to the ER at 37°C in a dose dependent manner (Figure 5B-lanes 4, 6, and 8). This may indicate that the G170D allele is mutant even at the permissive temperature for some functions of Hsp90. This increase in DES binding in the presence of 4-OHT in the *G170D* mutant was not observed in yeast cells that did not express the ER, indicating that it was dependent upon the presence of the receptor (Figure 6). In order to determine whether this phenotype was specific for the G170D mutant, 4-OHT competition assays were performed on six other *hsp82* mutants. These mutants can be grouped into two categories; 1) temperature sensitive mutants which include *A97I* (Kimura et. al., 1994), *T101I* (Kimura et. al., 1994), *G313N* (Bohen and Yamamoto, 1993), *S485Y* (Kimura et. al., 1994) and *T525I* (Bohen and Yamamoto, 1993), and 2) generalized mutants which are mutant at both temperatures, *E431K* (Bohen and Yamamoto, 1993). Of these mutants, *A97I* and *T101I* fall into the region of Hsp90 that is able to interact with the benzaquinoid ansamycin, geldanamycin (for schematic see Figure 7). The other mutants including *G313N*, *E431K*, *S485Y* and *T525I* are in the region of Hsp90 that is known to interact with steroid hormone receptors. Interestingly, in certain *hsp82* mutants there was very little increase in DES binding at 25°C, but the effect was amplified at 37°C; this group included *G170D* (1.2 fold increase at 25°C (Figure 5A and Figure 8A); 7.5 fold increase at 37°C (Figure 5B and 8B) and *G313N* (1.8 fold increase at 25°C (Figure 8A); 14 fold increase at 37°C) (Figure 8B). All *hsp82* mutants with the exception of *S485Y*

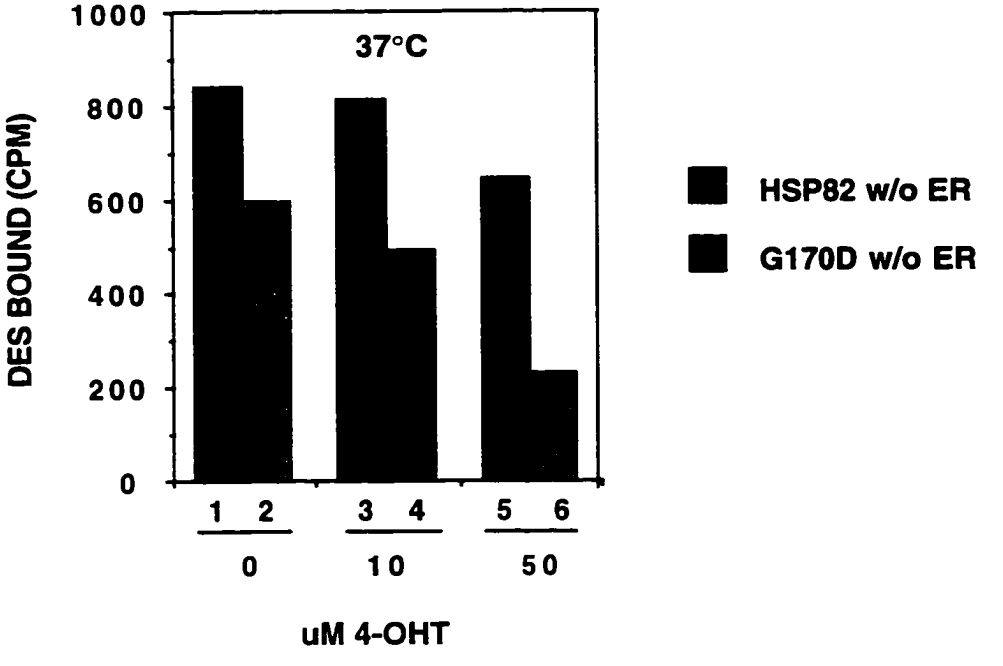


Figure 6. **4-OHT competition assay with DES in the absence of ER in wild type and *hsp82*^{G170D} mutant yeast**. Wild type strain AFY43 (*HSP82*; black bars; lanes 1,3, and 5) and *hsp82*^{G170D} (*G170D*; gray bars; lanes 2,4 and 6) mutant yeast strain AFY44 (without ER) were incubated at 37°C with varying amounts (0 μM (lanes 1 and 2), 10 μM (lanes 3 and 4) and 50 μM (lanes 5 and 6)) of 4-OHT in the presence of 100 nM [^{3}H] DES. Results are expressed as [^{3}H] DES bound (cpm x 10^{-2}).

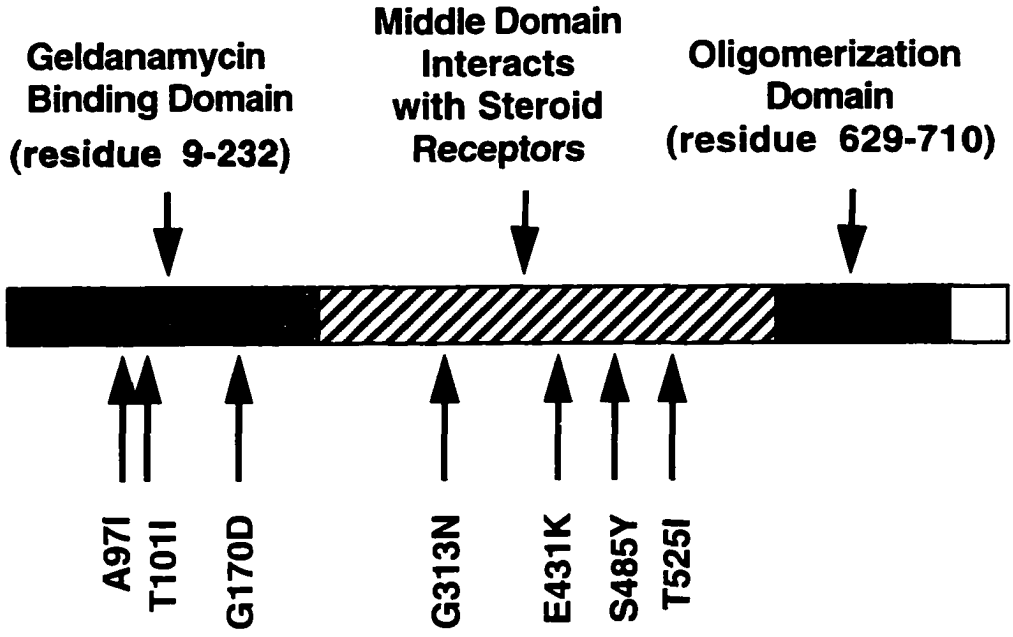


Figure 7. **Domain Structure of Hsp90.**

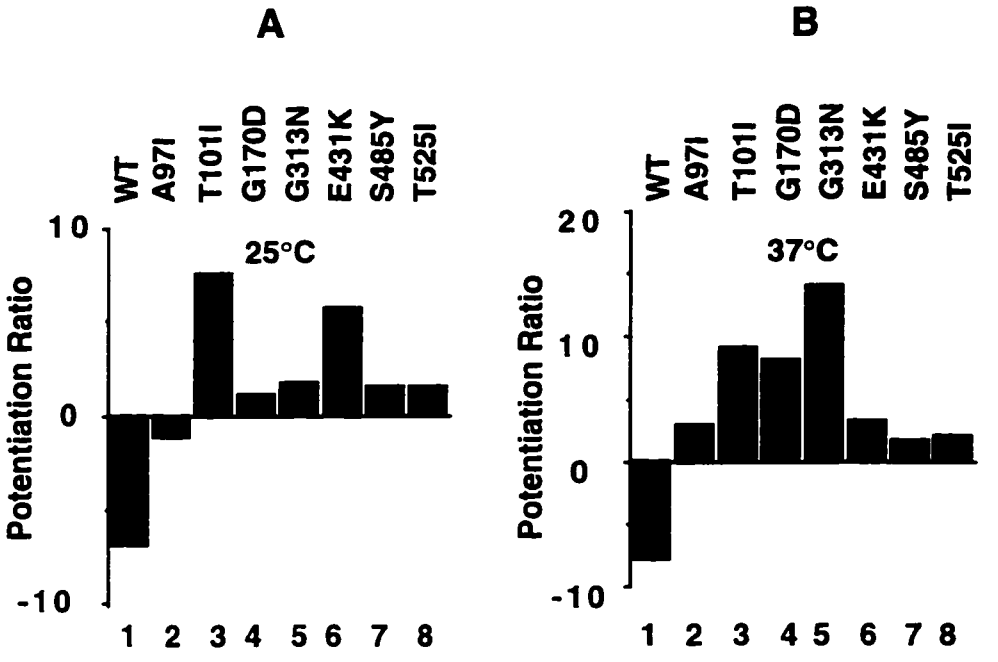


Figure 8. **4-OHT competition assays on various *hsp82* mutants**. (A and B) Wild type strain AFY43 (WT) and *hsp82* mutant (*A97I* (AFYA97IER), *T101I* (AFYT101IER), *G170D* (AFY43), *G313N* (AFYG313NER), *E431K* (AFYE431KER), *S485Y* (AFYS485YER), *and T525I* (AFYT525IER)) containing yeast heterologously expressing ER were incubated at 25°C (A) or 37°C (B) with and without 50 μM 4-OHT in the presence of 100 nM [^{3}H] DES. Results are expressed as Fold Potentiation (cpm [^{3}H] DES bound in the presence of 4-OHT divided by cpm [^{3}H] DES bound in the absence of 4-OHT).Results are the mean of three independent experiments.

demonstrated an increase in DES binding upon 4-OHT treatment (Figure 8A and B). In addition to the *G170D* mutant, the *G313N* and the *A97I* mutant were also decreased in their ability to bind DES at both the permissive and non-permissive temperature (Figure 9). All mutants and wild type yeast with the exception of *G313N* also contained similar quantities of Hsp90 protein as demonstrated by Western blot analysis (Figure 10). The level of Hsp90 protein was significantly decreased in the G313N mutant when compared to all other mutant and wild type strains tested (Figure 10). Thus, 4-OHT is able to increase binding of DES to the ER to differing extents in 6 different *hsp82* mutants. In order to determine whether the phenotype of the *hsp82* mutant yeast could be complemented with the wild type Hsp90, 4-OHT competition assays were performed on heterozygotes that contain both *HSC82* wild type and an *hsp82* mutant allele. No increase in DES binding upon 4-OHT treatment was seen in any of the heterozygotes tested, they all acted as wild type for 4-OHT competition (Figure 11). When these same heterozygotes were plated on 5-fluoro-orotic acid **(FOA)** (in order to deselect for the wild type *HSP82* plasmid), the mutant 4-OHT phenotype was restored. This indicates that the *hsp82* mutants tested are recessive and can be complemented by wild type *HSP82*. In order to determine whether this phenotype was specific for DES, 4-OHT competition assays were performed with ^{3}H- 17β-estradiol **(E2),** instead of DES. A similar increase in hormone binding upon 4-OHT treatment of the mutant was also observed when E2 was used as a ligand (Figure 12). In this case, however, there was a reduced signal to noise ratio resulting in

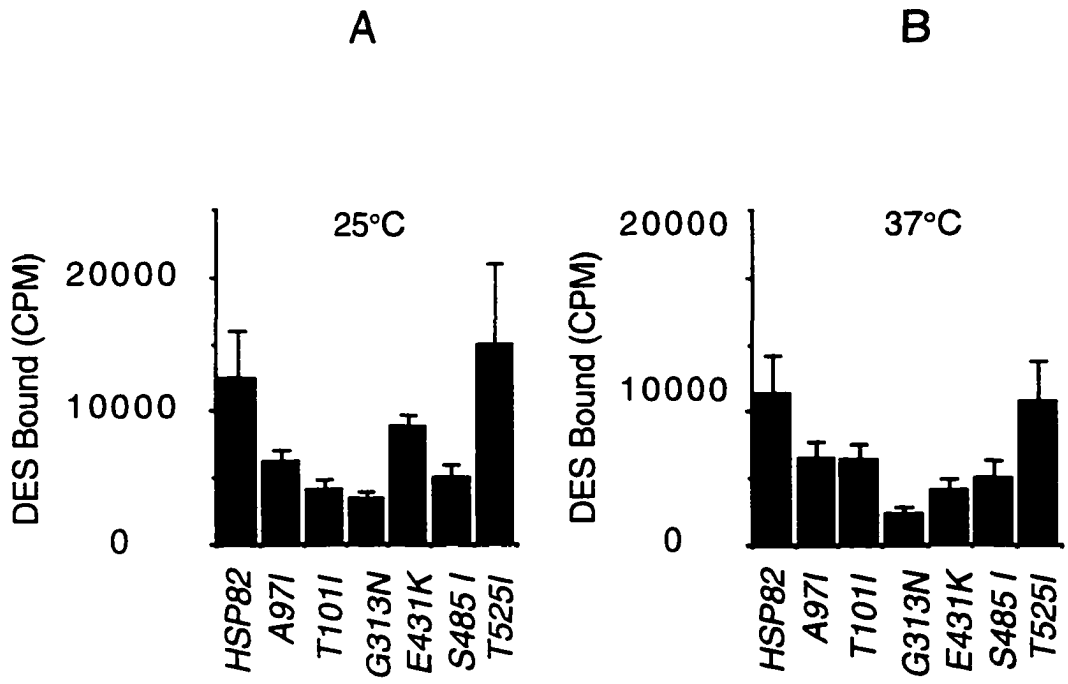


Figure 9. **Hormone binding to ER heterologously expressed in wild type and *hsp82* mutant yeast.** Wild type AFY43 (*HSP82*; lane 1) and AFYA971ER (*A971*, lane 2), AFYT1011ER (*T1011*, lane 3), AFYG313NER (*G313N*, lane 4), AFYE431KER (*E431K*, lane 5), AFYS485YER (*S485Y*, lane 6) and AFYT525IER (*T525I*, lane 7) mutant yeast heterologously expressing ER were incubated at 25°C (A) or 37°C (B) with 100 nM [^{3}H] DES. Results are expressed as [^{3}H] DES bound (cpm). Results are the mean of at least three independent experiments.

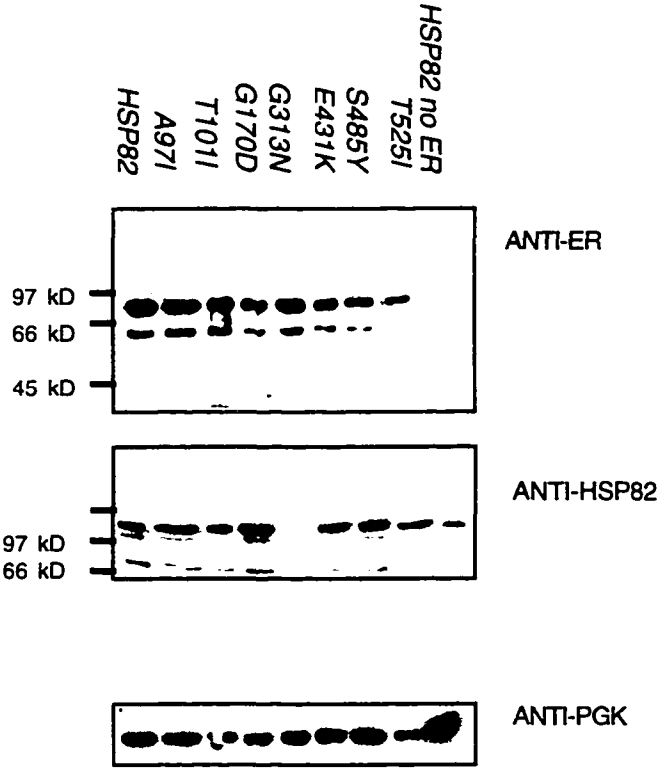


Figure 10. **Western blot analysis of ER in wild type and *hsp82* mutant yeast.** Western blot analysis of ER (top panel; arrow denotes location of ER and star denotes breakdown product or ER), Hsp90 (middle panel; arrow denotes location of Hsp90). Analysis was performed on whole cell extracts of wild type AFY43 (WT; lane 1), AFYA97IER (*A97I*; lane 2), AFYT101IER (*T101I*; lane 3), AFY44 (*G170D*; lane 4), AFYG313NER (*G313N*; lane 5), AFYE431KER (*E431K*; lane 6), AFYS485YER (*S485Y*; lane 7), AFYT525IER (*T525I*; lane 8) expressing ER and p82a (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).

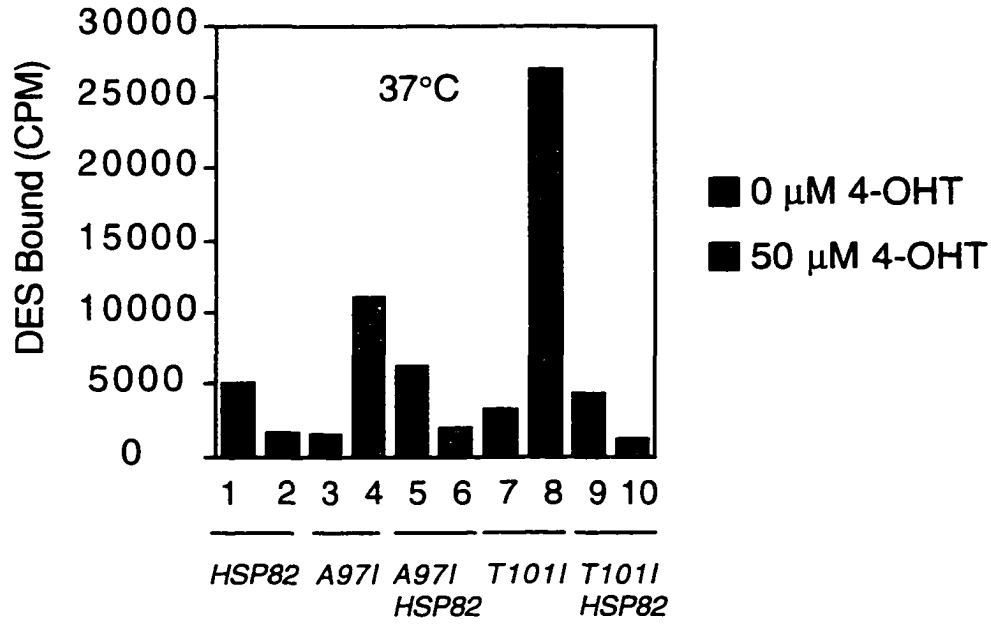


Figure 11. **4-OHT competition assays in a heterozygote *hsp82/HSP82* yeast strains.** Wild type AFY43 (*HSP82* , lanes 1 and 2), AFYA97IER (*A97I* , lanes 3 and 4), AFYA97IERHSP82 (*A97I/HSP82*, lanes 5 and 6), AFYT101IER(*T101I*, lanes 7 and 8) and AFYA97IERHSP82(*T101I/HSP82*, lanes 9 and 10) mutant yeast heterologously expressing ER were incubated at 37°C with (gray bars; lanes 2, 4, 6, 8 and 10) or without (black bars; lanes 1, 3, 5, 7 and 9) 50 µM 4-OHT in the presence of 100 nM [^{3}H] DES. Results are expressed as [^{3}H] DES (cpm).

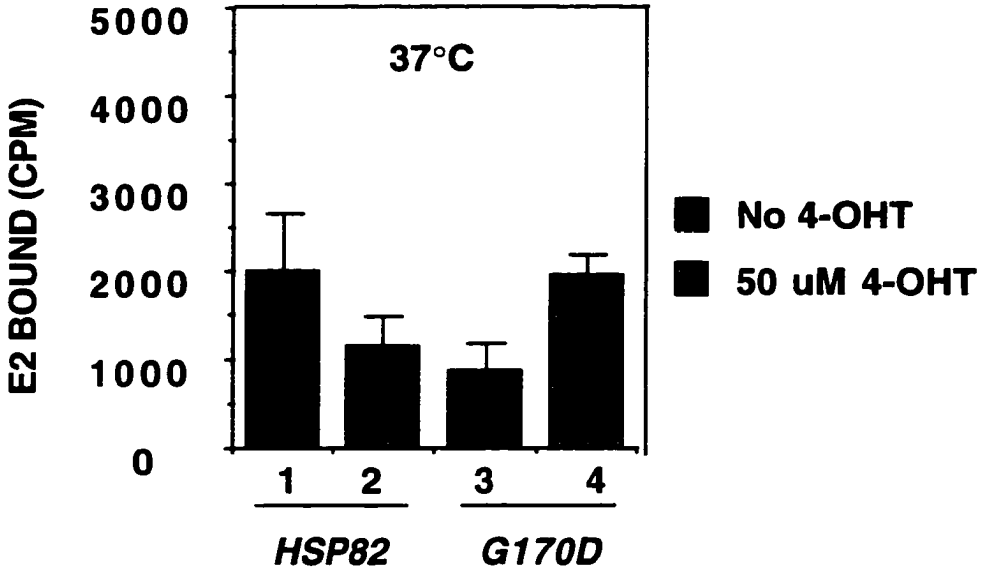


Figure 12. **4-OHT competition assay with E2 in wild type and *hsp82*^{G170D} mutant yeast.** Wild type AFY43 (*HSP82*) and AFY44 (*G170D*) mutant yeast heterologously expressing ER were incubated at 37°C with (gray bars; lanes 2 and 4) or without (black bars; lanes 1 and 3) 50 μM 4-OHT in the presence of 100 nM [^{3}H] 17ß-estradiol. Results are expressed as [^{3}H] 17ß-estradiol bound (cpm).

decreased levels of potentiation at 37°C (2 fold) (Figure 12-lane 4). To further investigate the 4-OHT induced increase in DES binding, the ability of 4-OHT to prevent DES dissociation from the ER in the *G170D* mutant was evaluated. This was performed by a two-step procedure whereby the ^{3}H-DES was incubated with the *G170D* cells in the presence of 4-OHT at both 25°C and 37°C. Subsequently, these cells were washed in media and were then re-incubated at 37°C in media in the presence or absence of 4-OHT. As shown in Fig. 13, cells that were subsequently incubated in media containing 4-OHT retained approximately 40% of the initially bound ^{3}H-DES (lane 2), but only if they were originally labeled at 37°C. If the cells were subsequently incubated in media lacking 4-OHT, the ^{3}H-DES that remained bound to the ER was only 15% of the original value (lane 1). This indicates that 4-OHT is able to prevent bound DES from dissociating. However, if the original binding was at 25°C, then 4-OHT could not prevent DES dissociation (lane 4). These data reflect upon the qualitative difference in the nature of ^{3}H-DES binding to the ER at the permissive and restrictive temperatures in the *G170D* mutant.

As described earlier previous studies have demonstrated that GR (Picard et. al., 1990), ER (Picard et. al., 1990), PR (Bohen and Yamamoto, 1993), AR (Fang et. al., 1996), MR (Picard et. al., 1990) and RAR (Holley and Yamamoto, 1995) are unable to fully transactivate upon hormone binding in the absence of functional Hsp90. Initial experiments were performed to determine if ligand dependent transactivation was also defective in the *G170D* mutant strain. As shown in Figure 14, there was no difference in the ability of

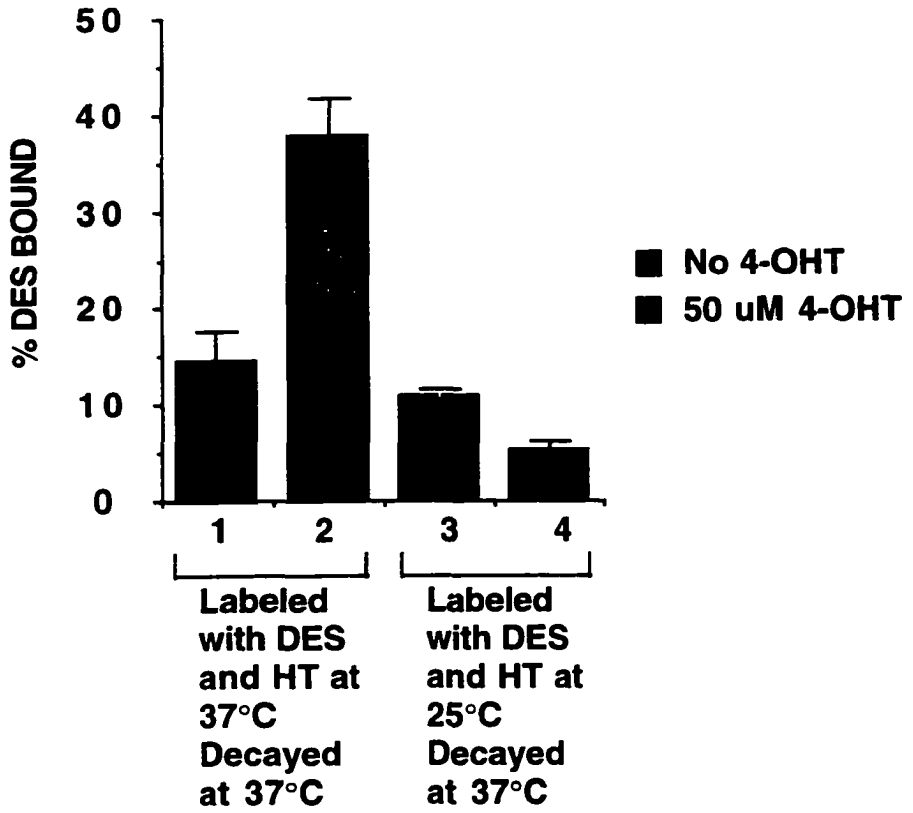


Figure 13. **Temperature shift hormone dissociation assay.** *hsp82*^{G170D} mutant yeast (AFY44) were incubated at either 25°C (lanes 1 and 2) or 37°C (lanes 3 and 4) with (lanes 1 and 3; black bars) or without (lanes 2 and 4; gray bars) 50 μM 4-OHT in the presence of 100 nM [^{3}H] DES. Subsequently all samples were washed and incubated at 37°C for 30 minutes. Results are expressed at % [^{3}H] DES bound. Results are the mean of three independent experiments.

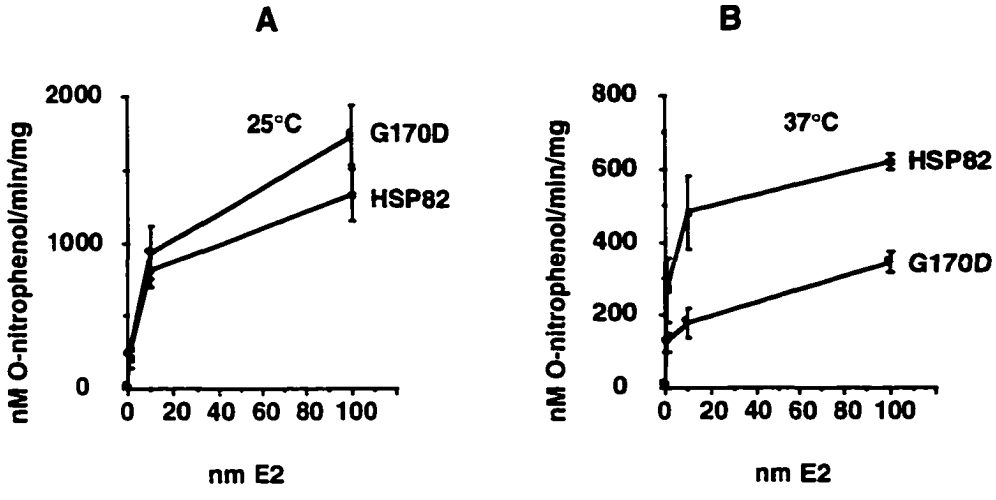


Figure 14. **E2 dependent transactivation by ER in Wild type and *hsp82*^{G170D} mutant yeast.** Wild type AFY43 (*HSP82*) and *hsp82*^{G170D} (AFY44) mutant yeast (*G170D*) which were heterologously expressing ER were incubated at 25°C (A) or 37°C (B) with increasing concentrations of E2 (0 nM-100 nM). Following the above treatments, whole cell extracts were made and ß-galactosidase activity was determined and expressed as nM O-nitrophenol/min/mg. Results are the mean of three independent experiments.

E2 to induce lacZ gene expression at the permissive temperature of 25°C (Figure 14A), whereas, 100 nM E2 stimulated lacZ gene expression in the wild type two fold over that of the *G170D* mutant at the non-permissive temperature of 37°C (Figure 14B). Previous studies have also demonstrated that 4-OHT was able to stimulate ER driven transactivation in the yeast (Lyttle et. al., 1992). Similarly, 4-OHT was able to stimulate lacZ gene expression in our yeast system (Figure 15). In order to determine whether this activity of 4-OHT was dependent upon Hsp90, lacZ reporter gene assays were performed on both wild type and G170D mutant yeast treated with 4-OHT. As seen in Figure 15, there was no difference in the ability of 4-OHT to stimulate lacZ gene expression at the permissive temperature of 25°C (Figure 15A), however, there was approximately a 2 fold decrease in 4-OHT driven transactivation in the mutant at the non-permissive temperature of 37°C when compared to the wild type strain (Figure 15B). To see whether DES bound to the ER in the presence of 4-OHT in the *G170D* mutant could stimulate transcription of a lacZ reporter gene, I analyzed the ability of the ER to transactivate the lacZ gene in wild type and *G170D* mutant cells that had been treated with DES, DES plus 4-OHT or 4-OHT by itself. As shown in Fig. 16, DES (100nM) stimulated lacZ gene expression 54-fold above the background in the wild type cells (lane 1), and 15-fold in the *G170D* mutant cells (lane 4) at the restrictive temperature. By itself, 4-OHT (50 μM) stimulated lacZ gene expression by 22-fold in the wild type (lane 2) and 4-fold in the mutant (lane 5). Thus, both ligands have a reduced ability to stimulate lacZ gene expression in the mutant compared to

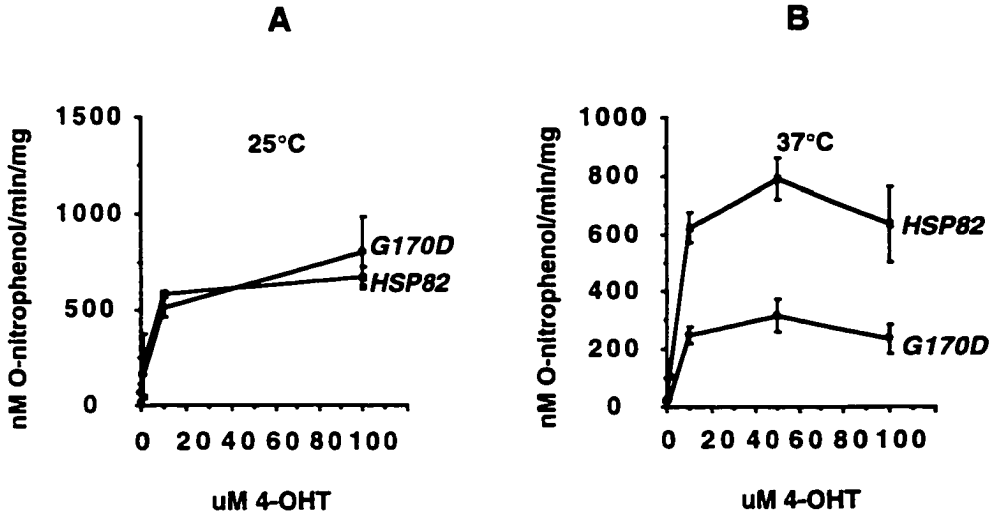


Figure 15. **4-OHT dependent transactivation by ER in Wild type and *hsp82*^{G170D} mutant yeast**. Wild type AFY43 (*HSP82*) and *hsp82*^{G170D} mutant yeast (AFY44) (*G170D*) expressing ER were incubated at 25°C (A) or 37°C (B) with increasing concentrations of 4-OHT (0 µM-100 µM). Following the above treatments, whole cell extracts were made and ß-galactosidase activity was determined and expressed as nM O-nitrophenol/min/mg. Results are the mean of three independent experiments.

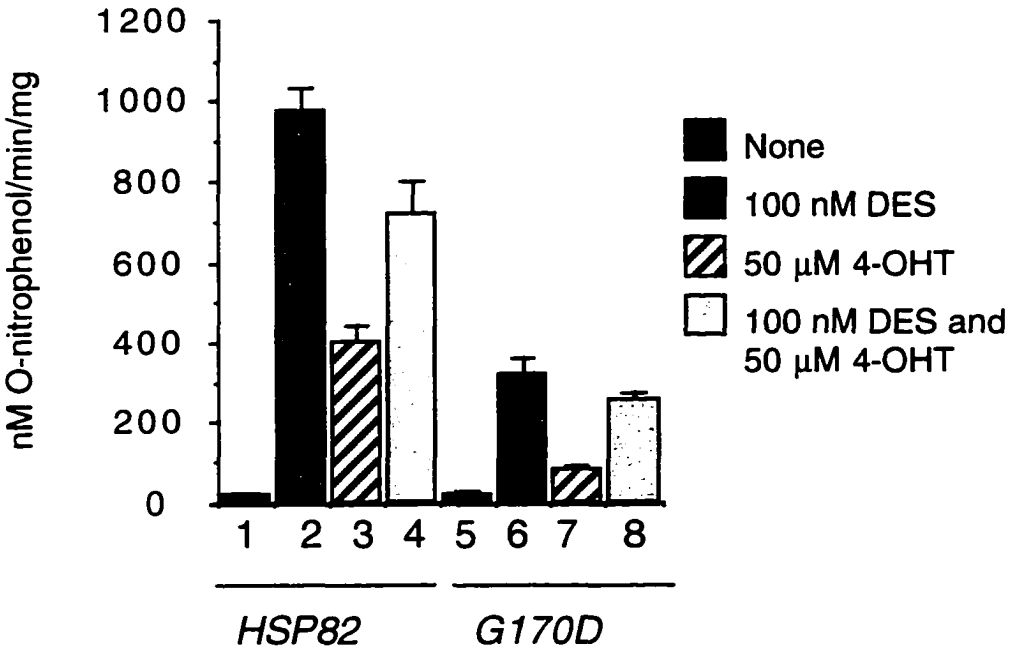


Figure 16. **Transactivation by ER in Wild type and *hsp82*^{G170D} mutant yeast.** Wild type AFY43 (WT; lanes 1-4) and *hsp82*^{G170D} mutant yeast (AFY44) (*G170D*; lanes 5-8) were incubated at 37°C with no treatment (lanes 1 and 5), 100 nM DES (lanes 2 and 6), 50 µM 4-OHT (lanes 3 and 7) or a combination of 100 nM DES and 50 µM 4-OHT. Following the above treatments, whole cell extracts were made and ß-galactosidase activity was determined and expressed as nM O-nitrophenol/min/mg. Results are the mean of three independent experiments.

the wild type. Upon mixing both ligands, there was a 40-fold stimulation of lacZ gene expression in the wild type (lane 3) and 12-fold in the mutant (lane 6). These data indicate that those receptors which have a 4-OHT induced increase in DES binding do not act as wild type with respect to transactivation. Furthermore, these data also demonstrate that 4-OHT does not substantially decrease lacZ gene expression induced by DES alone in the wild type cells, even though 4-OHT can compete for DES binding and stimulate receptor mediated transactivation in the wild type yeast.

[3] Hormone Binding to the AR is Defective in an Hsp90 Mutant Yeast strain

Previous *in vivo* studies in yeast have demonstrated that the *hsp82*^{G170D} was defective in hormone induced activation of the AR (Fang et al, 1996). This activation defect was maximal at subsaturating hormone concentrations, suggesting that the defect may be caused by the inability of the AR to bind hormone at high affinity in this mutant strain. However, Nemoto et. al. suggests that there is no change in high affinity hormone binding to the AR with the loss of Hsp90 function (Nemoto et al, 1992). However, these studies were performed *in vitro* at 4°C with a truncated form of the AR and their physiological relevance is uncertain. The goal of the following experiments is to determine whether the defect in AR signaling in the Hsp90 mutant is caused at least in part by a defect in high affinity hormone binding.

In vivo direct hormone binding assays were performed with live yeast in order to determine whether this defect in AR activation was caused by a deficiency in hormone binding. Both wild type and *hsp82*^{G170D} temperature sensitive mutant yeast that heterologously express human AR were assayed for there ability to bind ^{3}H R1881 methyltrielone **(R1881),** a synthetic androgen. In order to determine the level of background binding, direct hormone binding assays were performed with yeast that were not transformed with the human AR plasmid. Background binding in the yeast was negligible.

The results of the direct hormone binding assays are shown in Figure 17. At the permissive temperature of 25°C, AR in both wild type and *hsp82*^{G170D} yeast strains had similar binding isotherms and were equally able to bind R1881 (Figure 17-open symbols). These data suggest that the *hsp82*^{G170D} strain is phenotypically wild type for high affinity hormone binding to the AR at the permissive temperature. When similar hormone binding assays were performed at 37°C, the non-permissive temperature for the *hsp82*^{G170D} strain, there was a clear difference in the ability of the human AR to bind R1881 when comparing the wild type and *hsp82*^{G170D} strains (Figure 17-filled symbols). AR in the *hsp82*^{G170D} strain were decreased in their ability to bind R1881 when compared to the isogenic wild type strain. This difference was maximal at subsaturating concentrations of R1881 (Figure 17 (inset)), but was still apparent at higher hormone concentrations (Figure 17). The decrease in hormone binding seen in the mutant strain did not result from a change in steady state levels or relative stability of the AR (Fang et al, 1996).

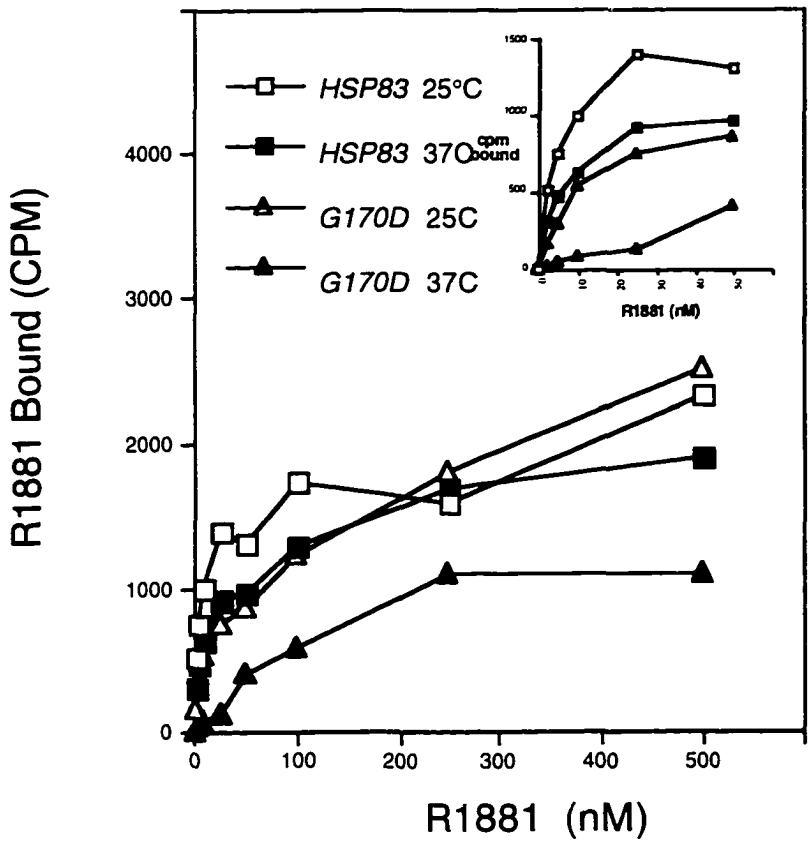


FIGURE 17. **Binding curve for AR in the wild type and *G170D* mutant yeast .** Titration of ^{3}H R1881 with wild type ACY98 and *hsp82*^{G170D} mutant yeast (ACY99) cells expressing AR at 25°C and 37°C as shown. Inset, enlarged view of ^{3}H R1881 titration from 0-50 nM. Legend as follows; open squares (*HSP82* at 25°C), filled squares (*HSP82* at 37°C), filled triangles (*G170D* at 37°C), open triangles (*G170D* at 25°C). Each data point is the mean of 3-5 independent experiments.

In order to determine the AR hormone binding affinity, the data from the direct hormone binding assays was analyzed according to the method of Scatchard. An estimate of the relative K_d was calculated from the Scatchard plot. As expected, there was no significant difference in the relative K_d's of the high affinity hormone binding state of the AR when comparing the wild type and *hsp82*^{G170D} mutant strains at the permissive temperature (Figure 18A and C). At the permissive temperature, the relative K_d's for AR in the wild type and mutant strains were 0.8 nM and 2.1 nM, respectively. Similarly at 37°C, the relative K_d for AR in the wild type strain was 1.0 nM (Figure 18B), whereas, the AR in the *hsp82*^{G170D} mutant strain were unable to bind hormone with high affinity at the non-permissive temperature (Figure 18D). AR in the mutant strain only had a low affinity binding state similar to that seen for the wild type at both temperature and *hsp82*^{G170D} yeast strains at the permissive temperature.

Earlier in this Chapter it was shown that there was an alteration in the ability of the drug 4-OHT to interact with the ER in several *hsp82* mutant yeast strains. The aim of the following experiments is to determine if Hsp90 loss of function alters the ability of the hormone antagonist HF to act as a competitive inhibitor of hormone binding to the AR.

Antagonist competition assays were performed with heterologously expressed AR in both the wild type and *hsp82*^{G170D} mutant strains. In these competition assays, live yeast cells were incubated with 100 nM ^{3}H R1881

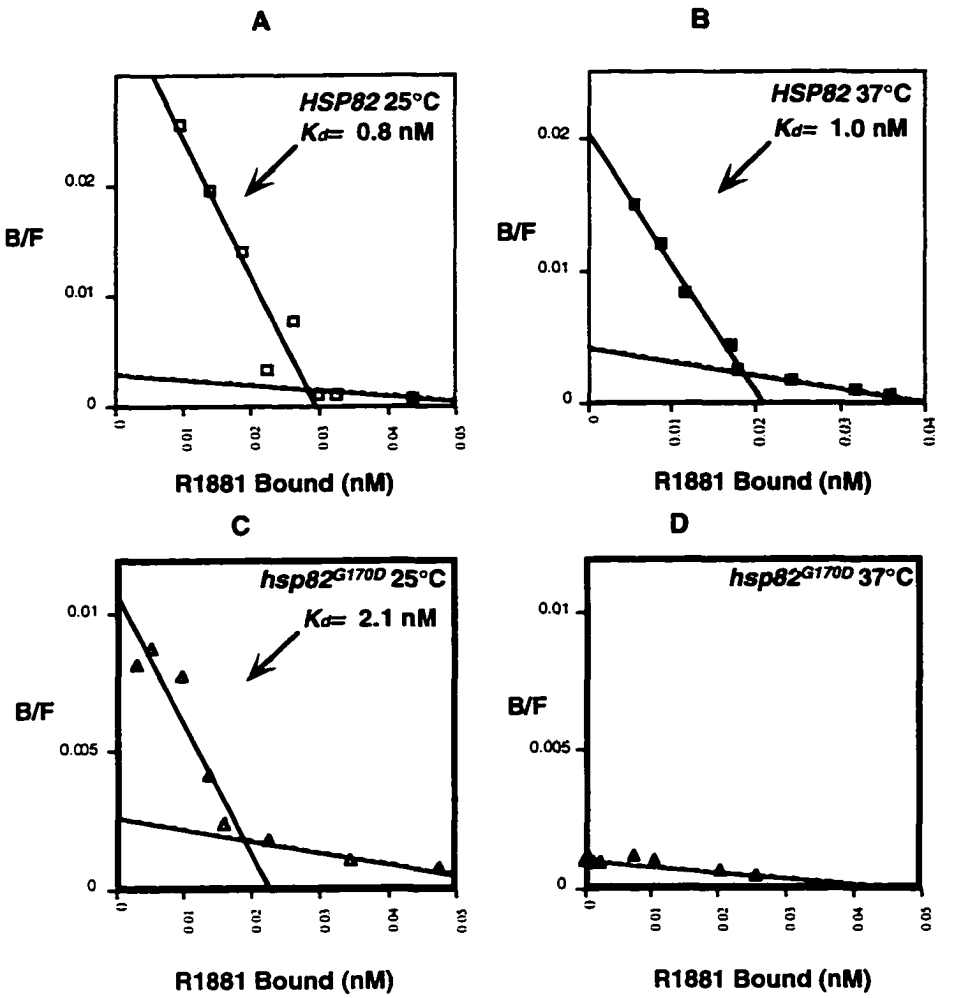


FIGURE 18. **Scatchard plot for AR in the wild type and *hsp82* mutant yeast.** A, Scatchard analysis of data from WT strain ACY98 at 25°C. B, Scatchard analysis of data from WT ACY98 strain at 37°C. C, Scatchard analysis of data from *hsp82* mutant strain ACY99 at 25°C. D, Scatchard analysis of data from *hsp82* mutant strain ACY99 at 37°C which were heterologously expressing AR. Each data point is the mean of 3-5 independent experiments. Legend as follows; open squares (WT at 25°C), filled squares (WT at 37°C), filled triangles (*hsp82*^{G170D} at 37°C), open triangles (*hsp82*^{G170D} at 25°C). Each data point is the mean of 3-5 independent experiments

and increasing amounts of the unlabeled HF. The results from these HF competition assays are shown in Figure 19. No significant difference was seen in the ability of HF to compete for R1881 binding to AR when comparing the wild type and *hsp82*^{G170D} strains at the permissive temperature (Figure 19A and B-open symbols). This was not unexpected since AR in the *hsp82*^{G170D} strain were wild type for hormone binding at the permissive temperature (Figure 17C). In both the *hsp82*^{G170D} and wild type strains, a 250 fold excess of HF reduced R1881 binding to about 70% of the original value. However, at the non-permissive temperature of 37°C, HF was able to compete for R1881 binding to a greater extent in the *hsp82*^{G170D} yeast strain when compared to the wild type strain (Figure 19B-closed symbols). At 25 μM HF in the *hsp82*^{G170D} strain, the remaining R1881 binding was reduced to approximately 20% of the original value, whereas, in the wild type strain R1881 binding was only reduced to 75% of the original value (Figure 19B-filled symbols).

Discussion

There has been renewed interest in the study of the function of Hsp90 since it was demonstrated to be the target for a group of anti-tumor agents called benzoquinoid ansamycins (Whitesell et. al., 1994). Recently, the amino-terminal domain of Hsp90 has been crystallized at high resolution in the presence of the benzaquinoid ansamycin, geldanamycin (Stebbins et. al., 1997). The crystal structure reveals that geldanamycin binds to the ATP binding domain of Hsp90. In addition to the geldanamycin binding domain,

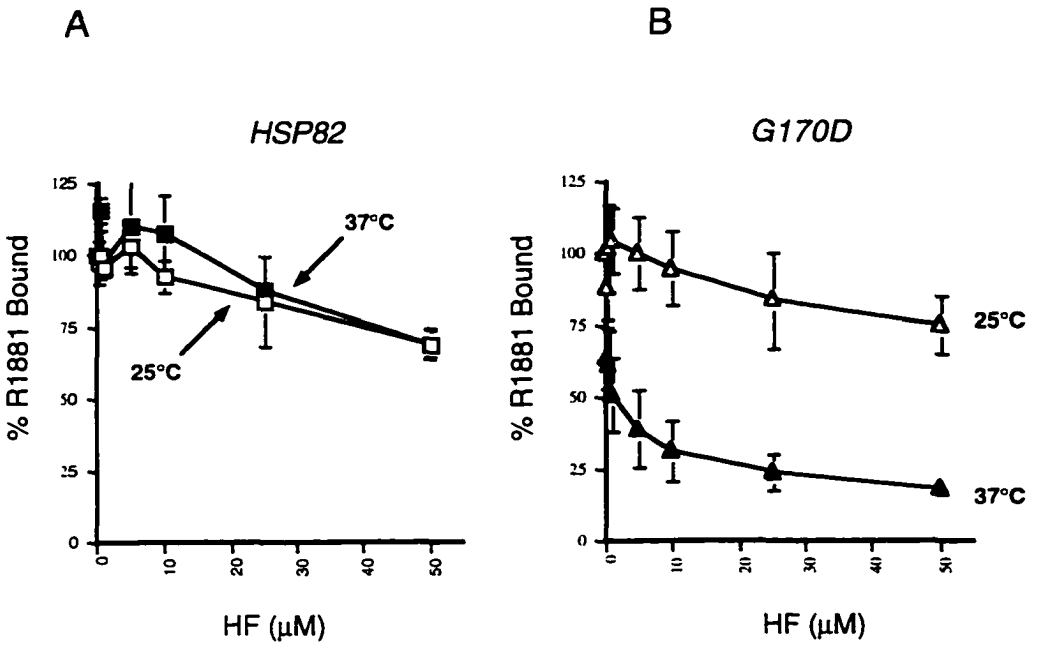


FIGURE 19. **HF competition assay in wild type and *G170D* mutant yeast expressing AR.** Wild Type ACY98 (A, *HSP82*) and *hsp82* mutant ACY99 (B, *G170D*) yeast strains were incubated with 100 nM ^{3}H R1881 and 0-50 μM HF for 1.5 hours at 25°C or 37°C. Specific binding was determined and plotted as a percentage of the counts from samples incubated with 100 nM ^{3}H R1881 in the absence of HF. Each point represents the mean of three independent experiments. Legend as follows; open squares (*HSP82* at 25°C), filled squares (*HSP82* at 37°C), filled triangles (*G170D* at 37°C), open triangles (*G170D* at 25°C). Each data point is the mean of 3-5 independent experiments.

Hsp90 contains a carboxy-terminal oligomerization domain and a middle domain which is known to interact with steroid hormone receptors (see Figure 7 for schematic of Hsp90 domain structure). *In vivo* studies performed using the yeast system have demonstrated that alterations in residues in either the geldanamycin binding or middle domain of Hsp90 cause a decrease hormone dependent steroid receptor activation of GR (Nathan and Lindquist, 1995; Bohen and Yamamoto, 1993; Bohen, 1995).

In this study it was demonstrated that mutations in the gene encoding Hsp90 affect the ability of the AR and ER to bind ligand. First, mutations in Hsp90 lead to a decrease in the ability of AR to bind R1881 and for ER to bind DES. In the *hsp82* mutant strains, AR was unable to bind R1881 to the same extent as in the isogenic wild type strain. Similarly in the *hsp82* mutant strain, ER was unable to bind DES to the same extent as in the isogenic wild type strain. Second, mutations in Hsp90 also affected the ability of hormone antagonists to compete for hormone binding to both the AR and ER. For the AR, HF was converted from a poor to a potent competitor of hormone binding to the AR upon loss of Hsp90 function. Likewise, the ability of the drug 4-OHT to compete for DES and E2 binding was altered. In several *hsp82* mutant strains 4-OHT increased hormone binding to the ER. This suggests that Hsp90 plays an important role in the folding of the AR and ER to a hormone binding state.

Hsp90 binds to unliganded ER (Reduluih et. al., 1987; Segnitz and Gehring, 1995; Ratajczak et. al., 1987; Schlatter et. al., 1992; Ratajczak et. al.,

1990) , although there is very little information on the physiological relevance of this interaction, since results from previous *in vitro* studies suggest that Hsp90 is not required for hormone binding (Binart et. al., 1995; Aumais et. al., 1997). Our results, however, are consistent with a role for Hsp90 in maintaining the ER in the high affinity hormone binding state *in vivo*, since in the absence of a functional Hsp90 the ER has a decreased ability to bind DES (Figure 4B). Ligand binding studies were initially performed with ^{3}H E2, but it was found that there was a high background of non-specific binding in the yeast, as originally described by Burshell et. al. (1984) and Lyttle et. al. (1992). This was due to the presence of a previously described E2 binding protein in the yeast. This problem was overcome with the use of ^{3}H DES, instead of ^{3}H E2. Recently, Segnitz and Gehring also suggested that hormone binding to the ER may be compromised in live cells treated with geldanamycin, a benzoquinoid ansamycin that specifically inhibits the function of Hsp90 (1997). Both of these results suggest that Hsp90 may play an important role in ER hormone binding *in vivo*.

Previous studies have shown that 4-OHT competes for hormone binding to the ER (Capony and Rochefort, 1978). This is in sharp contrast to the results presented here for the ER, in which 4-OHT was able to increase DES binding to the ER in the absence of a functional Hsp90 (Figure 5B). Just how does 4-OHT stimulate DES binding to the ER? In order for a protein to be reach its native state presumably it must pass through a series of folding intermediates. As with most reaction intermediate, these conformers are usually transient in

nature and are normally not seen under physiological conditions. I postulate that the increase in DES binding by 4-OHT is a consequence of the partial folding of the ER to an folding intermediate which is present in *hsp82* mutant strains. This intermediate is able to bind 4-OHT in some way that increases DES binding in the absence of a functional Hsp90. It is also possible that distinct folding intermediates may exist in different *hsp82* mutants, since differences were seen in the ability of 4-OHT to increase DES binding in the various *hsp82* mutants tested (Figure 8). For example, in the *G170D* mutant, 4-OHT did not significantly increase DES binding (1.2 fold increase) to the ER at 25°C (Figure 5A and 8A), but neither did it compete for this binding. At 37°C, however, 4-OHT increased DES binding to the receptor in G170D by 7.5 fold (Figure 5B and 8B) . This suggests that the ER may adopt more than one intermediate folding state in *hsp82* mutant strains. This mutant phenotype was fully complemented upon transformation of the mutant strains with a wild type *HSP82* plasmid as demonstrated with the heterozygote strains, suggesting that these Hsp90 mutation are recessive in nature (Figure 11). One clue to the mechanism of this phenotype was that the largest 4-OHT induced increase in DES binding (14 fold) was observed in the *G313N* mutant at 37°C (Figure 8B), which also had the least amount of mutant Hsp90 protein as detected by Western blot (Figure 10). This suggests that the 4-OHT induced increase in DES binding phenotype may occur in ER molecules that are not in direct association with Hsp90 and that the formation of this folding intermediate is dependent upon other chaperones.

Previous studies have shown that Hsp90 is required for steroid hormone receptors to transactivate target genes in yeast, and that mutations in or depletion of Hsp90 reduce this activity (Picard et. al., 1990; Bohen and Yamamoto, 1993; Bohen, 1995; Nathan and Lindquist, 1995; Fang et. al., 1996). In this study, I also find that mutation in Hsp90 results in a reduced ability of the ER to transactivate a target lacZ gene (Figure 14-16). As shown in Fig. 14 and 16, at the non-permissive temperature the transactivation potential of DES (Figure 16) and estradiol (Figure 14) is reduced in the *G170D* mutant strain compared with the wild type. Similar results are also seen for 4-OHT, which stimulates transactivation by the ER in yeast (Figure 15- experiments performed by Jie Rao). Taken together, these results suggest that Hsp90 is also required for ligand dependent transactivation by ER. Interestingly, the increase in DES binding by 4-OHT did not lead to a subsequent increase in transactivation in the mutant (Figure 16). This suggests that under these conditions the ER is in a conformation distinct from the active state. This could reflect the receptor having both ligands bound simultaneously to the same monomer, or by having them bound one to each monomer in a heterodimer or even a mixed population of both. Likewise, this 4-OHT stimulated increase in DES binding may not be accompanied by the proper conformational changes to allow for downstream transactivation. Alternatively, Hsp90 may have an additional role in ER action distinct or downstream of ligand binding.

Similar to the ER, Hsp90 binds to the unliganded state of the AR, however, there is little known on its role in AR activation. Previous in vitro studies suggest that AR is able to bind hormone in the absence of Hsp90 (Nemoto et. al., 1992). However, these studies were performed at 4°C with a truncated form of AR and can not rule out a role for Hsp90 in hormone binding to the AR. The results presented here, however, are consistent with a role for Hsp90 in maintaining the AR in a high affinity binding conformation, since in the absence of functional Hsp90 the AR is reverted to low affinity binding conformation (Figure 17-19). Under wild type conditions, there were two distinct binding states of the AR (a high and low affinity binding state), however, upon Hsp90 loss of function the high affinity binding state disappeared (Figure 18). Thus, the Hsp90 requirement for AR seems to be somewhat similar to that of PR, since at low temperature, PR is able to bind hormone in the absence of Hsp90, however, when experiments were performed at higher temperature (37°C) Hsp90 is required for hormone binding (Smith, 1993). This is In contrast to the GR, which is unable to bind hormone in the absence of Hsp90 under all conditions (Bresnick et. al., 1989).

Earlier in this chapter it was shown that there was a concurrent alteration in the ability of a hormone antagonist 4-OHT to compete for hormone binding to the ER with loss of Hsp90 function. In order to determine whether this was the case with the AR, ligand competition assays were performed on wild type and Hsp90 mutant yeast which were heterologously expressing the AR. Previous studies have shown that the AR antagonist HF is able to bind to the AR and

compete for hormone binding, but the affinity of this interaction is approximately 50 fold less than that of dihydrotestosterone (DHT) (Veldscholte et. al., 1992; Wong et. al., 1995). Thus, under normal conditions HF is a poor competitor of hormone binding to the AR. The results presented here for the wild type yeast confirm this observation (Figure 19A). In the presence of wild type Hsp90, a 500 fold excess of HF was only able to reduce R1881 binding to 70-80% of the original value (Figure 19A). However, upon Hsp90 loss of function, HF was able to acts as a more potent competitor of R1881 binding (Figure 19B). Under these loss of function conditions, HF was able to reduce R1881 binding to approximately 20% of its original value (Figure 19B-closed symbols). These data demonstrate a concurrent alteration in the ability of HF to compete for hormone binding to the AR. This alteration is quite different from the one seen with 4-OHT and the ER. Instead of potentiating hormone binding as 4-OHT did with DES binding to the ER, HF was made a more potent competitor in the loss Hsp90 function. This suggests that Hsp90 plays slightly different roles in the regulation of hormone binding when comparing the AR and ER, possibly revealing an inherent difference in their requirement for molecular chaperones. These differences may in fact be in the types and stability of folding intermediates formed in the pathway to the maturation of the AR and ER. Taken together the studies presented here demonstrate that Hsp90 plays an important role in the attainment or maintenance of hormone binding to both the AR and ER.

Experimental Methods

Materials

DHT, E2, DES and 4-OHT were obtained from Sigma Chemical Company. R1881 was purchased from NEN. The above compounds were solubilized in ethanol and stored at -20°C. Antisera to ER was a kind gift of Dr. G. Greene. Antisera to Hsp90 and AR was previously described (Fang et. al., 1996). Antisera to yeast phosphoglycerate kinase was a kind gift of Dr. P. Lazarow. ^{3}H DES, ^{3}H E2 and ^{3}H R1881 were purchased from American Radiochemical Company, NEN, and NEN respectively. Isogenic wild type and *G170D* mutant yeast strains were a kind gift of Dr. S. Lindquist. Plasmids encoding the *A97I* (pts38RV), *T101I* (pcs2-3RV) and *S485Y* (pts33BE) *hsp82* mutants were kind gift of Dr. Y. Kimura and those encoding *E431K* (pTCA/hsp82 E431K), *G313N* (pTCA/hsp82 G313N) and *T525I* (pTCA/hsp82 T525I) were kind gift of Dr. K. Yamamoto.

Plasmid Constructions

Plasmids that express the AR (pARU and pARH) were derived from pG1-hAR as described by Caplan et al. (1995). Briefly, pARU was constructed by inserting a blunt-ended URA3 fragment into EcoRV-linearized pG1-hAR (inside 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. (Purvis et. al., 1991). The human ER open reading frame from p2HGPDER/CYC (Picard et. al., 1990) was subcloned into the vector pRS424 (Christianson et. al., 1992). Briefly, 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.

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 (Geitz et. al., 1995).

The *hsp82* mutant strains (except for *G170D* which was the gift of Dr. S. Lindquist) were constructed from strain p82a (Nathan and Lindquist, 1995) as previously described (Fang et al., 1998). This strain was transformed with

pJR1 (Fang et al., 1998) (CEN6/ARS4/HSP82/URA3), and the resulting strain (AFY2W) was grown in rich medium to deselect for pTGPD (HSP82/TRP1). Resulting TRP auxotrophs were designated AFY1WU. This strain was then transformed with plasmids containing *hsp82* mutant genes (see below) and subsequently plated on 5-fluoro-orotic acid to select for loss of pJR1. The resulting strains all contained a single *hsp82* mutant allele. 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 (Picard et. al., 1990). Yeast strains containing *A97I* (pts38RV), *T101I* (pcs2-3RV) or *S485Y* (pts33BE) were transformed with the ER expression plasmid pJR3. The p82a and *G170D* yeast strains were transformed with both p2HGPDER/CYC (Picard et. al., 1990). and pUCΔSS-ERE (Picard et. al., 1990).

ß-Galactosidase Activity Assay

Yeast cells were grown to early log phase (OD^{600}= 0.2) and preincubated at either 25°C or 37°C for one hour prior to the addition of either 17ß-estradiol, DES, 4-OHT or a combination of DES and 4-OHT. These cells were then incubated for an additional 2 hours at the same temperature prior to harvesting and the preparation of extracts as previously described (Caplan et. al., 1995) ß-galactosidase activity assays were performed as previously described (Caplan et. al., 1995).

Ligand Binding Assays

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 minutes. Following this preincubation, cells were incubated with either ^{3}H R1881 for the AR or ^{3}H DES for the ER for an additional 1.5 hours at the same temperature. The cells were then washed 3 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 R1881 for the AR or DES for the ER from the samples incubated in the absence of cold R1881 or DES.

Ligand competition assays were also performed with yeast cells which were grown to early log phase (OD^{600}= 0.2) as described above. Following a 30 minute preincubation at either 25°C or 37°C, cells were incubated with 100 nM ^{3}H R1881 for the AR and either 100 nM ^{3}H DES or ^{3}H 17ß-estradiol for the ER in the presence or absence of increasing concentrations of HF for the AR and 4-OHT for the ER for an additional 1.5 hours at the same temperature. The cells were then washed 3 times with 1 ml of water each and counted in 5 ml of scintillation fluid.

Temperature shift DES dissociation assays were performed as follows. *G170D* cells were grown in selective media containing 2% glucose to early log phase (OD^{600}= 0.2) and were subsequently incubated at either 25°C or 37°C

for 30 minutes. Subsequently, yeast cells were incubated with 100 nM ^{3}H DES in the presence of 50 μM 4-OHT for 1.5 hours at the same temperature as the preincubation. The cells were then washed 3 times with an equal volume of media. Following the wash, an aliquot of cells was counted in 5 ml of liquid scintillation fluid. The remainder of the cells were incubated for an additional 30 minutes at either 25°C or 37 °C in fresh media plus or minus 10 μM DES or 50 μM 4-OHT. Aliquots were removed for counting as described above. CPM were represented at percent remaining bound DES compared to the initial aliquot counted.

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 (Caplan et. al., 1995) .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 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20 **(TTBS)** and blocked overnight at room temperature with TTBS containing 5% non-fat dry milk. Filters were subsequently incubated with antibodies specific for the ER or yeast Hsp90 (antibodies were diluted in antibody dilution buffer, 1x 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 hour (anti-Hsp90) or 4 hours (anti-ER).

Filters were washed three times for 10 minutes each in TTBS. Filters were then incubated with secondary antibody (Horseradish Peroxidase (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 hour and subsequently washed three times for 10 minutes 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 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.

CHAPTER III

CHARACTERIZATION OF THE ROLE OF YDJ1 IN ANDROGEN AND ESTROGEN RECEPTOR ACTION

Introduction

As previously described, the Hsp90 chaperone machine is comprised of multiple components including Hsp70 and its regulatory factors. Previous studies have demonstrated that Hsp70 is required for hormone binding to the GR and PR *in vitro* (Hutchison et. al., 1993; Smith et. al., 1992). Hutchison et. al. demonstrated that upon depletion of Hsp70, GR was unable to bind hormone and likewise the aporeceptor complex containing Hsp90 was not formed. One of the known co-chaperones that helps regulate the Hsp70 reaction cycle is E. coli DnaJ (Liberek et. al., 1991a) (see Figure 20 for Hsp70 reaction cycle). Ydj1 is the yeast homologue of DnaJ (Caplan et. al., 1991). Ydj1, like dnaJ, also stimulates the normally low level ATPase activity of Hsp70, which is required for Hsp70 to bind polypeptide substrates (Tsai and Douglas, 1996; Liberek et. al., 1991b). In its ATP bound form, Hsp70 is unable to stably bind and fold polypeptides, whereas, in its ADP bound form Hsp70 stably interacts with polypeptides. All J proteins contain a characteristic amino-terminal J domain (see for review Cheetham and Caplan, 1998). This characteristic J domain along with the glycine and phenylalanine rich region is responsible for the ability of J proteins to stimulate Hsp70's ATPase activity (Wall et al, 1994). Likewise, some J proteins also contain a zinc finger and carboxy-terminal domain which have chaperoning functions of their own (Lu and Cyr, 1997; Szabo et. al., 1995). Based on their domain structure, J

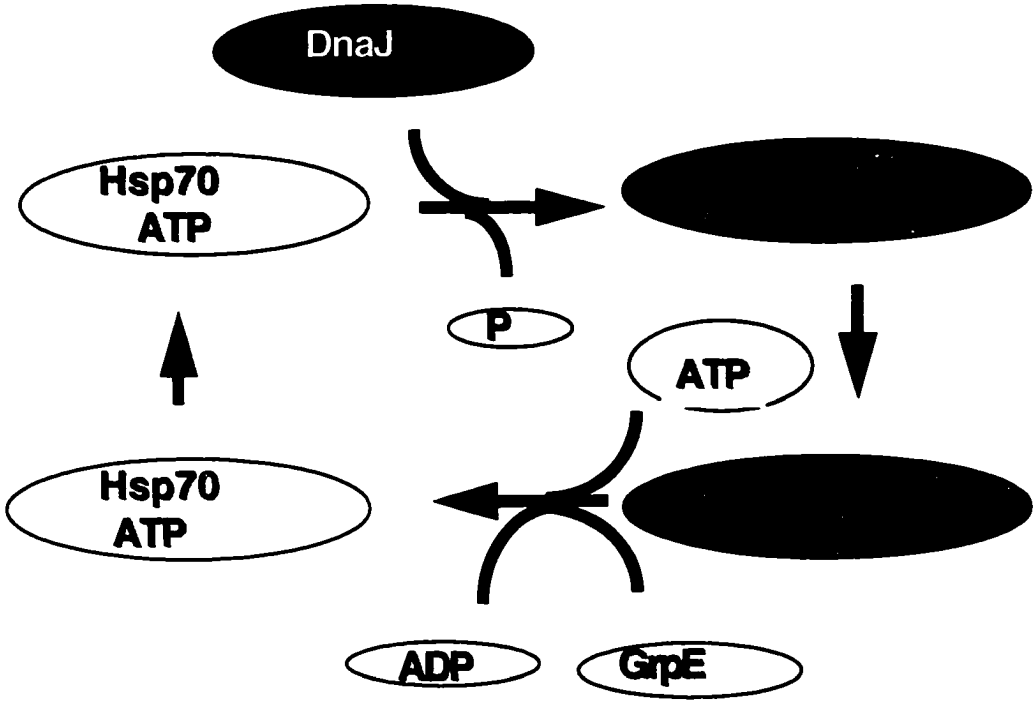


Figure 20. **Hsp70 Reaction Cycle**

proteins can be grouped into 3 main categories (see Figure 21); 1) Type 1 J proteins which contain the J, G/F and zinc finger domains and 2) Type2 J proteins which contains the JGF domain, but lacks the zinc finger domain and 3) Type 3 which contains only the J domain (See for review Cheetham and Caplan, 1998). Ydj1 and its human homologue Hdj2 are considered as Type 1 J proteins since they contain a zinc finger-like domain carboxy-terminal to the J G/F domain (see Figure 22) (Caplan et al, 1991; see review Cheetham and Caplan, 1998). Using the yeast model system, Caplan et al (1995) demonstrated that Ydj1 is required for the *in vivo* activation of AR, since hormone-dependent activation of the AR was decreased in yeast strains containing either a deletion of (*Δydj1*) or mutation in (*ydj1-151* which contains multiple point mutation throughout the coding sequence) the *YDJ1* allele. This activation defect was mediated via the HBD (Caplan et al, 1995). A similar study demonstrated that a yeast strain containing a $ydj1^{G315D}$ mutant allele (contains a mutation in the carboxyterminal domain) caused derepression of hormone induced ER and GR activation. In these experiments, yeast strains containing the $ydj1^{G315D}$ mutant allele had increased levels of hormone independent activity (Kimura et al, 1995). These data suggest a possible dual role for Ydj1 in steroid receptor activation.

In this study the yeast is used as a model system in order to determine the role that Ydj1 plays in hormone binding and activation of the AR and ER. A combination of hormone binding and ligand competition assays were utilized in an effort to determine which domains of Ydj1 are required for AR signaling

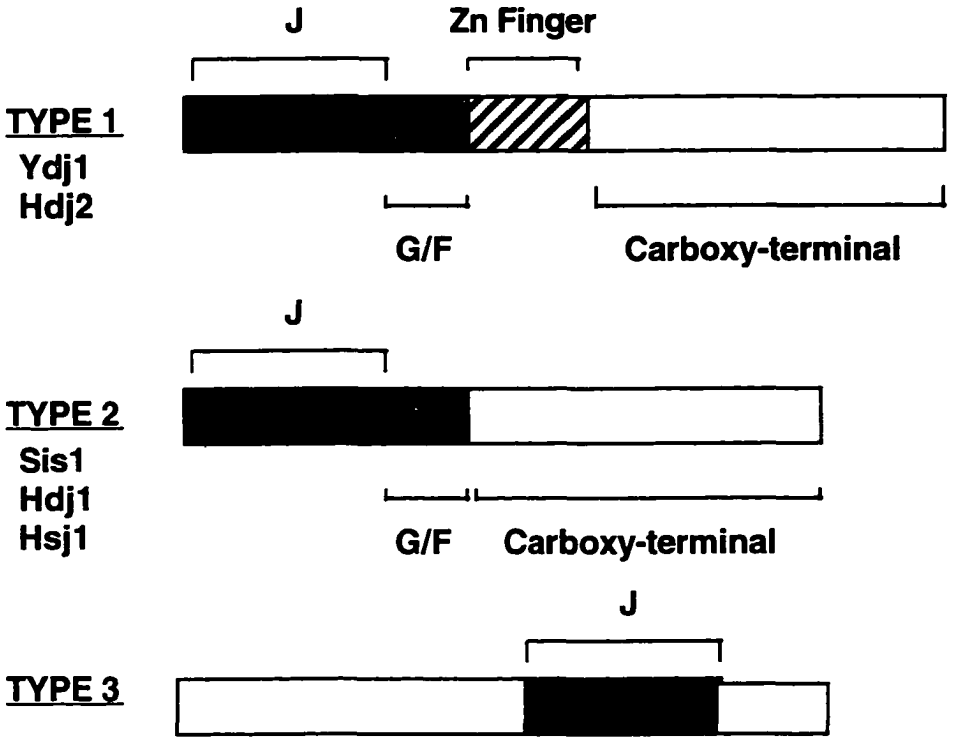


Figure 21. **Catergories of J Proteins**

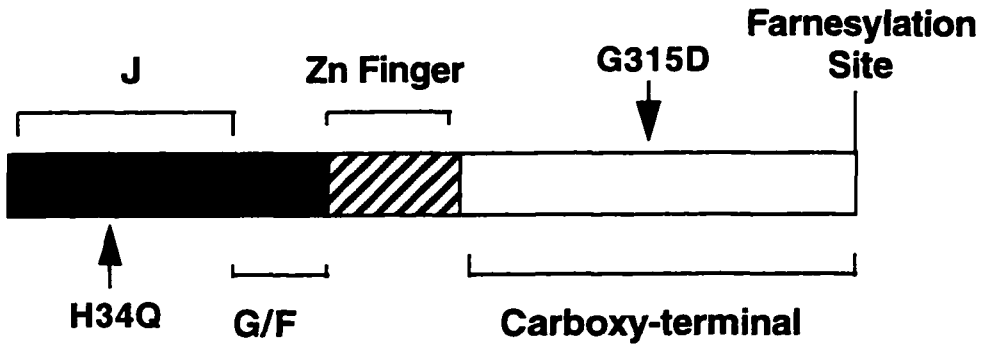


Figure 22. **Domain Structure of Ydj1**

activity. The results from these experiments suggest that Ydj1 is acting via the J and zinc finger domains to allow for wild type hormone binding to the AR and most probably ER. The results presented here also suggest that Ydj1 or more correctly its human counterpart (Hdj2) is required for high affinity hormone binding to both the AR and the ER.

Results

Previous studies have demonstrated that both AR and ER signaling are defective in yeast that contain a mutation or deletion of Ydj1p (Caplan et al., 1995; Kimura et al., 1996) and in the case of the AR this defect is mediated via the HBD. Therefore studies were initiated to test whether Ydj1 was required for hormone binding to the AR and ER. Ligand binding studies were performed on both wild type and ydj1 mutant yeast which had been transformed by a plasmid constitutively expressing either the AR or the ER.

[1] R1881 Binding to the AR is Defective in the Absence of Ydj1p

To test whether Ydj1 is required for hormone binding to the AR, direct hormone binding assays were performed on AR heterologously expressed in either the isogenic wild type and *Δydj1* mutant yeast. Initial studies were performed on a strain lacking Ydj1p (***Δydj1***). Loss of Ydj1p resulted in a temperature sensitive growth phenotype where the cells are viable, but slow growing at 25°C, and inviable at 37°C (Figure 23). AR expressed in both of these strain was able to specifically bind R1881. This specific binding was

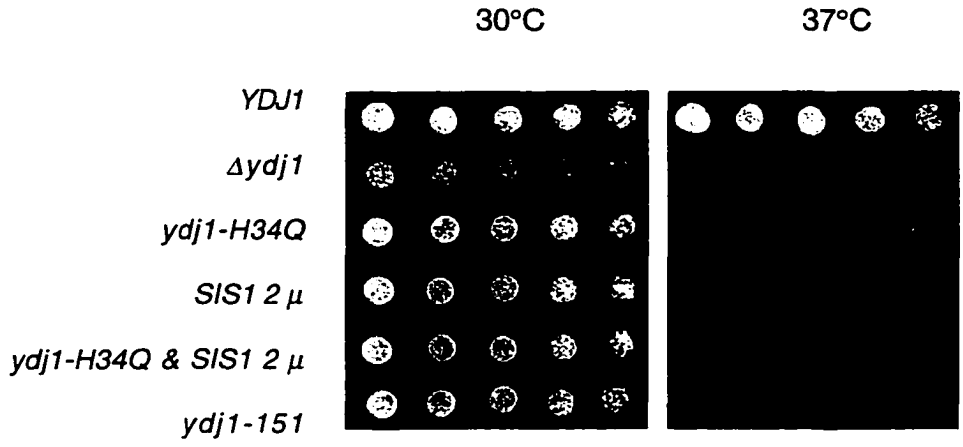


Figure 23. **Growth characteristics of the yeast *ydj 1* mutant strains**. Serial dilutions of yeast cells and growth at 30°C and 37°C. Wild type strain MYY290AR (*YDJ1*), *Δydj 1* strain AFY100AR (*Δydj 1*), *ydj 1-H34Q* in *Δydj 1* strain AFY100H34Q (*ydj 1-H34Q*), *SIS1* 2μ in *Δydj 1* strain AFY100SIS1 (*SIS1* 2μ), both *ydj 1-H34Q* and *SIS1* 2μ in *Δydj 1* strain AFY34Q/SIS (*ydj 1-H34Q/SIS1* 2μ) and *ydj 1-151* in *Δydj 1* strain AFY100-151 (*ydj 1-151*).

dependent upon the presence of the AR, since binding was negligible in the absence of the receptor. As seen in Fig. 24, there was a decrease in the ability of the AR to bind R1881 in the *Δydj1* strain at 25°C when compared to that of the isogenic wild type strain, even though receptor levels were similar as determined by Western blot analysis (Figure 25). This decrease in hormone binding was apparent at both saturating (3 fold decrease) and subsaturating (>6 fold decrease) levels of ^{3}H-R1881. This indicates that the AR is decreased in its ability to bind hormone in the absence of Ydj1p.

As demonstrated in Chapter 2, a concurrent alteration in the ability of HF to compete for hormone binding to the AR was seen in the absence of functional Hsp90. HF was a more potent competitor of R1881 binding to the AR with Hsp90 loss of function. In order to determine if there was a similar alteration in the ability of HF to compete for hormone binding in the absence of Ydj1p, ligand competition assays were performed on heterologously expressed AR in the both the wild type and *Δydj1* strains as previously described. In these competition assays, live yeast cells were incubated with a fixed amount of ^{3}H R1881 and increasing amounts of the unlabeled antagonist HF. The results are shown in Figure 26. As can be seen in Fig. 26, HF is a poor competitor of R1881 binding to the AR in the wild type strain at 25°C (filled triangles). In the wild type strain, a 250 fold excess of HF was only able to reduce R1881 binding to approximately 80% of its original value. In contrast, when the same experiments were performed with the *Δydj1* strain, similar concentrations of cold HF were able to reduce R1881 binding to approximately 30% of its

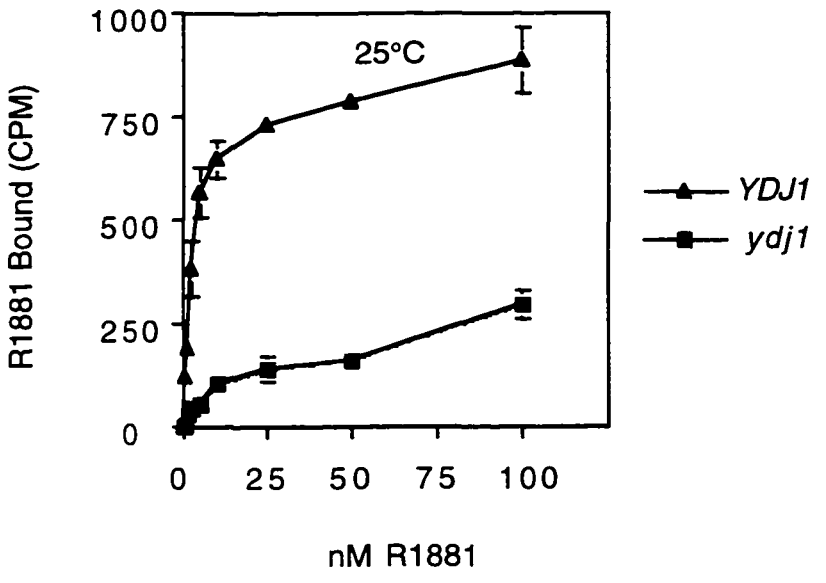


Figure 24. **Hormone binding to AR in wild type *YDJ1* and *Δydj 1* mutant yeast strains.** Titration of 3H-R1881 in yeast cells heterologously expressing AR. AR in wild-type strain MYY290AR (*YDJ1*; filled triangles) and *Δydj 1* mutant strain AFY100AR (*Δydj 1*; filled squares) were tested for R1881 binding at 25°C. Results are the mean of three independent experiments.

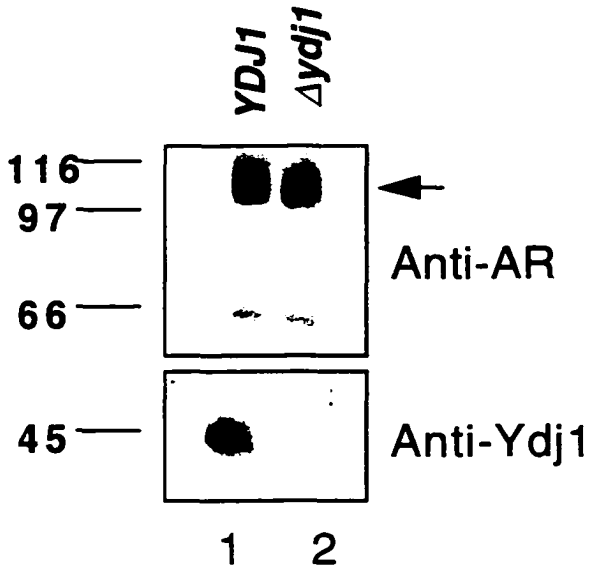


Figure 25. **Western blot analysis of AR in wild type and Δydj1 mutant yeast.** (top panel; arrow denotes location of AR and star denotes breakdown product or AR) and Ydj1 (lower panel; arrow denotes location of Ydj1). Analysis was performed on whole cell extracts of wild type strain MYY290AR (YDJ1; lane 1) and *Δydj1* strain AFY100AR (*Δydj1*; lane 2) yeast expressing AR probed with either antisera specific for AR (top panel) or Ydj1p (bottom panel). Molecular weight standards are shown in kDa.

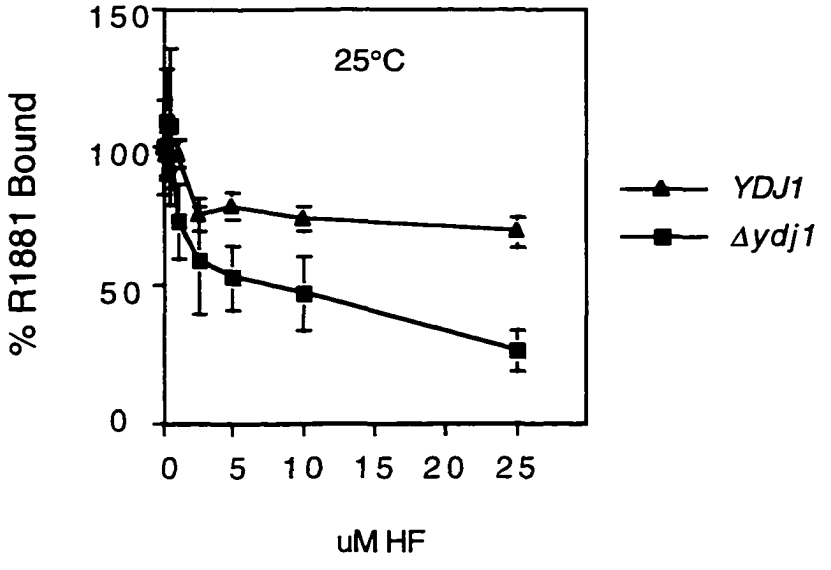


Figure 26. **HF competition assay on AR in wild type *YDJ1* and *Δydj1* mutant yeast.** Wild type strain MYY290AR (*YDJ1*; filled triangles) and *Δydj1* (*Δydj1*; filled squares) mutant strain AFY100AR heterologously expressing AR were incubated at 25°C with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

original value (Figure 26-closed squares). This indicates that the ability of HF to compete for hormone binding to the AR is altered in the absence of Ydj1p, similar to that seen for Hsp90 (Chapter 2).

[2] The J Domain, but not the Carboxy-terminal domain of Ydj1 is Required for Hormone Binding to the AR

Ydj1 and other Type 1 J proteins contain three major domains (see Figure 21 for schematic); 1) the conserved J G/F domain is responsible for stimulating the normally low level of ATPase activity of Hsp70 (Liberek et. al., 1991; Tsai and Douglas, 1996), 2) the zinc finger domain (which is only seen in Type 1 J proteins), and 3) the carboxy-terminal domain. Both the zinc finger and carboxy-terminal domains have chaperoning functions of their own (Lu and Cyr, 1997; Szabo et. al., 1995). In order to determine which domains of Ydj1 are required for ligand binding and whether these affects are via Hsp70, direct hormone binding and HF competition assays were performed on *Δydj1* yeast which were transformed with various yeast Ydj1 homologues and mutants (see Table 2 for list). The yeast J mutants and homologues are broken up into 4 major group according to their defect; 1) those that are defective in J domain function which include *ydj1-H34Q* (Lu and Cyr, 1998), *ydj1-39* (Dey et. al., 1996), 2) those that only contain the J/GF domain, but lack the zinc finger domain and farnesylation signal which includes SIS1, 3) those that are defective in the carboxy-terminal domain which includes *ydj1-G315D* (Kimura et. al., 1995), 4) those mutants that had combination defect including *ydj1-151*

TABLE 2: YDJ1 Homologue and Mutant Alleles

Yeast YDJ1 Homologues and Domains	
ydj1-151	contains 13 point mutations throughout the *YDJ1*
ydj1-H34Q	functional deletion of ATPase stimulatory domain
ydj1-G315D	mutation in carboxy-terminal domain
ydj1-39	contains 2 point mutations in J domain
SIS1	Yeast homologue of *dnaJ*
Human YDJ1 Homologues	
HDJ1	Human J homologue without Zn finger domain
HDJ2	Human J homologue of *YDJ1* (contains all domains)
HSJ1	Human J homologue without Zn finger domain

(Caplan et. al., 1995) and *ydj1-H34Q/SIS1* expressed in trans. As demonstrated in Fig. 23, transformation of the *Δydj1* strain with either *ydj1-H34Q, ydj1-151, SIS1* 2μ or *ydj1-H34Q/SIS1* trans were able to complement the slow growth at 25°C phenotype, but none complemented the temperature sensitive growth at 37°C. The results of the direct hormone binding assays for the J domain mutants are represented in Fig. 27 and 28. Yeast that contain either the *ydj1-H34Q* (Figure 27-open circles) or *ydj1-39* (Figure 28-closed squares) mutant alleles demonstrated a decrease in R1881 binding when the experiments were performed at 25°C, when compared to wild type yeast. Expression of *ydj1-H34Q* in the *Δydj1* parental strain was only able to partially complement (two fold increase over *Δydj1*) the hormone binding defect, indicating that the J domain is required to some extent for wild type hormone binding. Likewise, overexpression of SIS1 (contains J G/F domain, but lacks the zinc finger domain and farnesylation signal which are present in Ydj1p) in the Δydj1 strain was only able to partially complement the hormone binding defect (Figure 27-open triangles) (two fold over *Δydj1*). When experiments were performed with either ydj1-H34Q or SIS1 at 37°C a similar decrease in R1881 binding was observed (Figure 29A). In order to determine whether the J domain acts in *cis* or in *trans* with either the remainder of the molecule, direct hormone binding assays were performed on *Δydj1* yeast that had been transformed with both the *ydj1-H34Q* and 2 μ *SIS1* plasmid. As seen in Fig. 27, the expression of *ydj1-H34Q* and *SIS1* on separate plasmids was unable to fully complement the hormone binding defect exhibited in the *Δydj1* strain

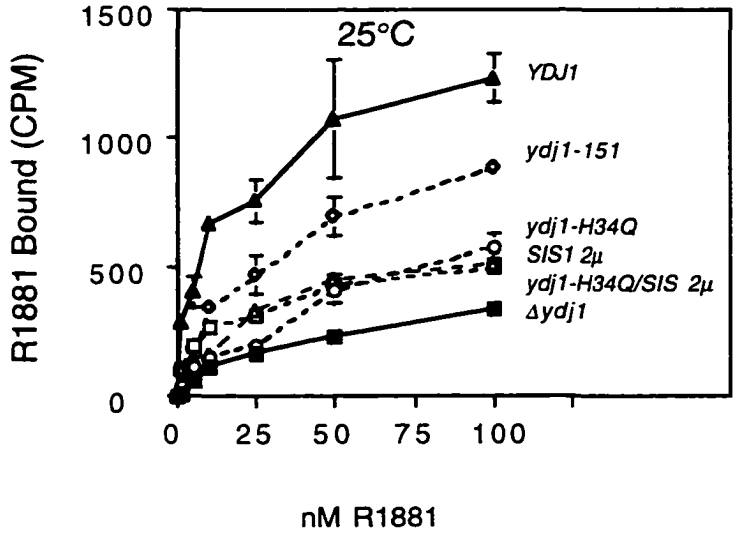


Figure 27 **Hormone binding to AR in *ydj 1* mutant yeast strains**. Titration of 3H-R1881 in *ydj 1* mutant yeast cells heterologously expressing AR. AR in *Δydj 1* mutant cells transformed with pAV7 strain AFYpAV7 (low copy number plasmid containing wild type *YDJ1*) (*YDJ1*; filled triangles), *Δydj 1* mutant strain AFY100AR (*Δydj 1*; filled squares), *Δydj 1* cells transformed with *ydj 1-H34Q* strain AFY100H34Q (low copy number plasmid containing *ydj 1-H34Q* mutant allele) (*ydj 1-H34Q* open circles), *Δydj 1* mutant yeast transformed with *SIS1* strain AFY100SIS1 (*SIS1*, 2μ plasmid) (*SIS1* 2μ; open triangles), *Δydj 1* mutant yeast transformed with both *ydj 1-H34Q* and *SIS1* strain AFY34Q/SIS (*ydj 1-H34Q/SIS1* 2μ; open squares) and *Δydj 1* transformed with *ydj 1-151* containing plasmid strain AFY100-151 (*ydj 1-151*; open diamonds) were tested for R1881 binding at 25°C. Results are the mean of three independent experiments.

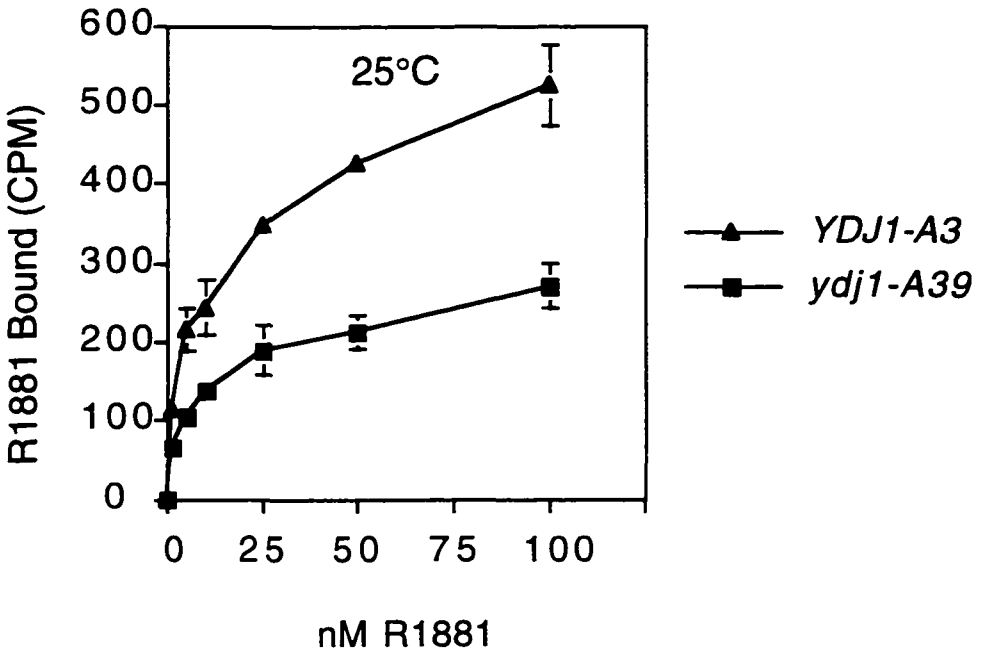


Figure 28. **Hormone binding to AR in wild type and *ydj1-39* mutant yeast strains.** Titration of 3H-R1881 in wild type and *ydj1-39* mutant yeast strains heterologously expressing AR. AR in wild type (*YDJ1-A3*; closed triangle) and *ydj1-39* mutant (*ydj1-A39*; closed squares) yeast cells were tested for R1881 binding at 25°C. Results are the mean of three independent experiments.

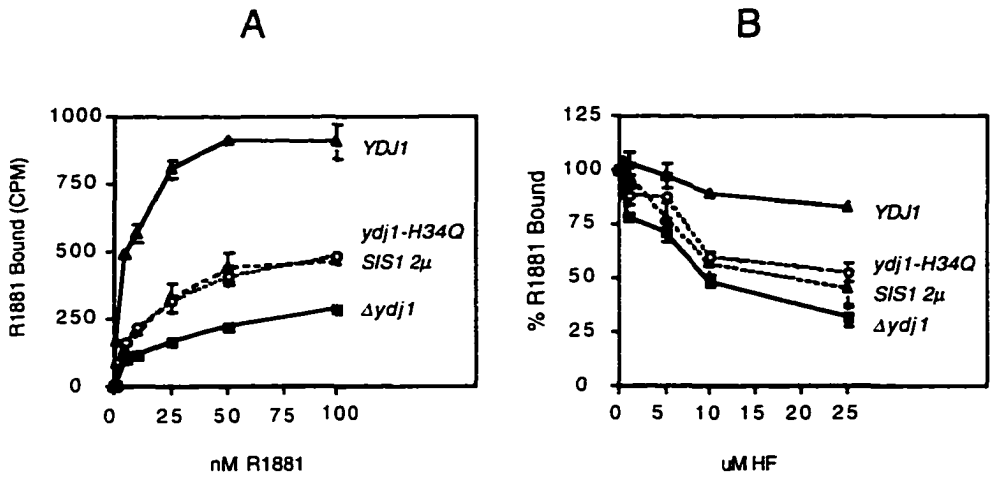


Figure 29. **Ligand binding to AR in *ydj 1* mutant yeast strains.** (A) Titration of 3H-R1881 in *ydj 1* mutant yeast cells heterologously expressing AR. AR in *Δydj 1* mutant cells transformed with pAV7 strain AFYpAV7(low copy number plasmid containing wild type *YDJ1)* (*YDJ1*; filled triangles), *Δydj 1* mutant strain AFY100AR (*Δydj 1*; filled squares), *Δydj 1* cells transformed with *ydj 1-H34Q* strain AFY100H34Q(low copy number plasmid containing *ydj 1-H34Q* mutant allele) (*ydj 1-H34Q* open circles), and *Δydj 1* mutant yeast transformed with *SIS1* strain AFY100SIS1(*SIS1*, 2μ plasmid) (*SIS1* 2μ; open triangles) were tested for R1881 binding at 37°C. Results are the mean of three independent experiments. (B) AR in *Δydj 1* mutant cells transformed with pAV7 strain AFYpAV7 (low copy number plasmid containing wild type *YDJ1*) (*YDJ1*; filled triangles), *Δydj 1* mutant strain AFY100AR (*Δydj 1*; filled squares), *Δydj 1* cells transformed with *ydj 1-H34Q* strain AFY100H34Q(low copy number plasmid containing *ydj 1-H34Q*mutant allele) (*ydj 1-H34Q*, open circles), and *Δydj 1* mutant yeast transformed with *SIS1* strain AFY100SIS1 (*SIS1*, 2μ plasmid) (*SIS1* 2μ; open triangles) were incubated at 37°C with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

(open squares), indicating that the J domain works in *cis* with the remainder of Ydj1 to allow for wild type hormone binding. In fact, expression of both SIS1 and ydj1-H34Q in *trans* was unable to increase hormone binding over that of each alone. Interestingly, expression of the *ydj1-151* (contains multiple point mutations in all domains with the exception of the J), which was previously demonstrated to be defective in AR signaling (Caplan et al., 1995), was able to almost fully complement the hormone binding defect of the *Δydj1* strain (Fig. 27-open triangles) (three fold increase over Δydj1). The only Ydj1 mutant or homologue that was not defective in hormone binding was the *ydj1-G315D* (Figure 30-closed squares), which contains a mutation in the conserved carboxy-terminal domain. R1881 binding in the *ydj1-G351D* strain was in fact somewhat higher than that of its isogenic wild type partner both at 25°C and 37°C, indicating that the carboxy-terminal domain is not neccessary for hormone binding to the AR.

In order to determine which domains are responsible for the increase in HF competition seen in the *Δydj1* strain, HF competition assays were performed on the same Ydj1 mutants and yeast homologues as with the direct hormone binding assays. Results for the HF competition assays are shown in Figure 31-33. Similar to the situation with direct hormone binding, neither *ydj1-H34Q* nor *SIS1* nor a combination of *ydj1-H34Q* and *SIS1* (Figure 31) nor *ydj1-39* (Figure 32) were able to fully restore HF competition to wild type levels with the exception of the *ydj1-151* mutant which was also essentially wild type for hormone binding as measured in the direct hormone binding assays Fig. 27

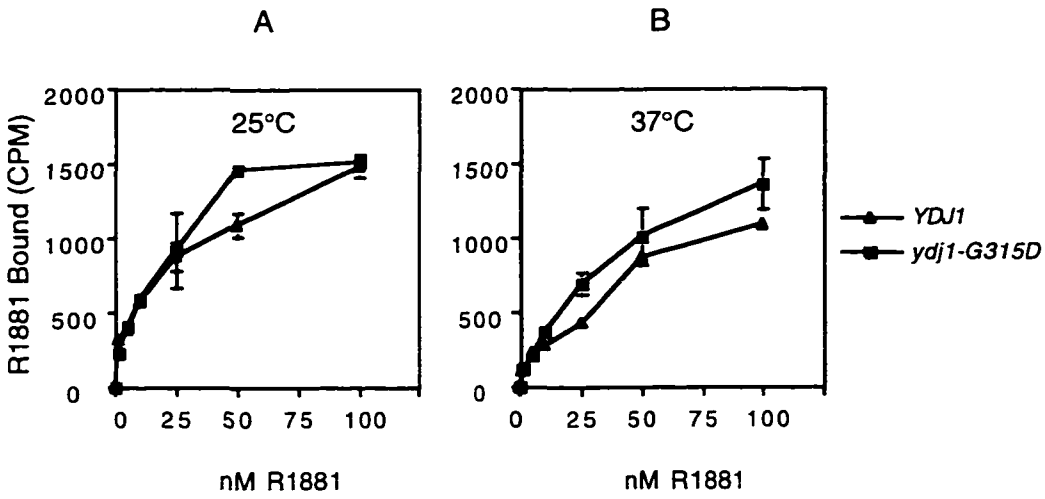


Figure 30. **Hormone binding to AR in wild type and *ydj1-G315D* mutant yeast strains.** Titration of 3H-R1881 in wild type and *ydj1-G315D* mutant yeast cells heterologously expressing AR. AR in wild type (*YDJ1*; closed triangle) and *ydj1-G315D* mutant (*ydj1-G315D*; closed squares) yeast cells were tested for R1881 binding at 25°C. Results are the mean of three independent experiments.

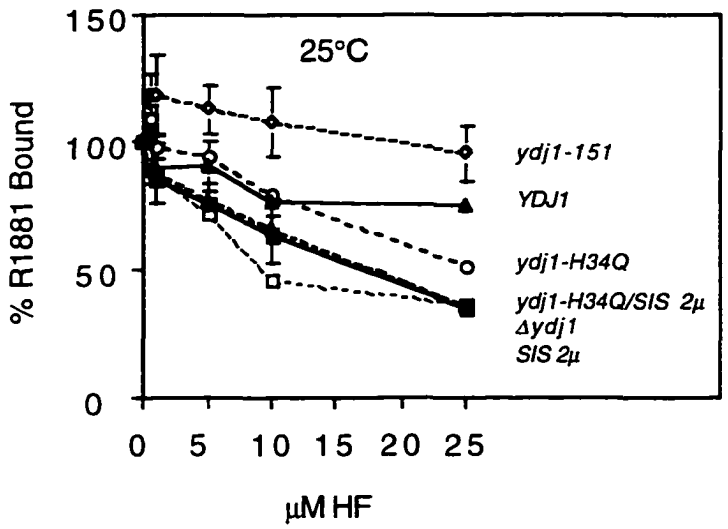


Figure 31. **HF competition assay on AR in *ydj 1* mutant yeast.** AR heterologously expressed in *Δydj 1* mutant cells transformed with pAV7 strain AFYpAV7 (low copy number plasmid containing wild type *YDJ1*) (*YDJ1*; filled triangles), *Δydj 1* mutant strain AFY100AR (*Δydj 1*; filled squares), *Δydj 1* cells transformed with *ydj 1-H34Q* strain AFY100H34Q (low copy number plasmid containing *ydj 1-H34Q* mutant allele) (*ydj 1-H34Q*, open circles), *Δydj 1* mutant yeast transformed with *SIS1* strain AFYSIS1 (*SIS1*, 2μ plasmid) (*SIS1* 2μ; open triangles), *Δydj 1* mutant yeast transformed with both *ydj 1-H34Q* and *SIS1* strain AFY34Q/SIS (*ydj 1-H34Q/SIS1* 2μ; open squares) and *Δydj 1* transformed with *ydj 1-151* containing plasmid strain AFY100-151 (*ydj 1-151*; open diamonds) were incubated at 25°C with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

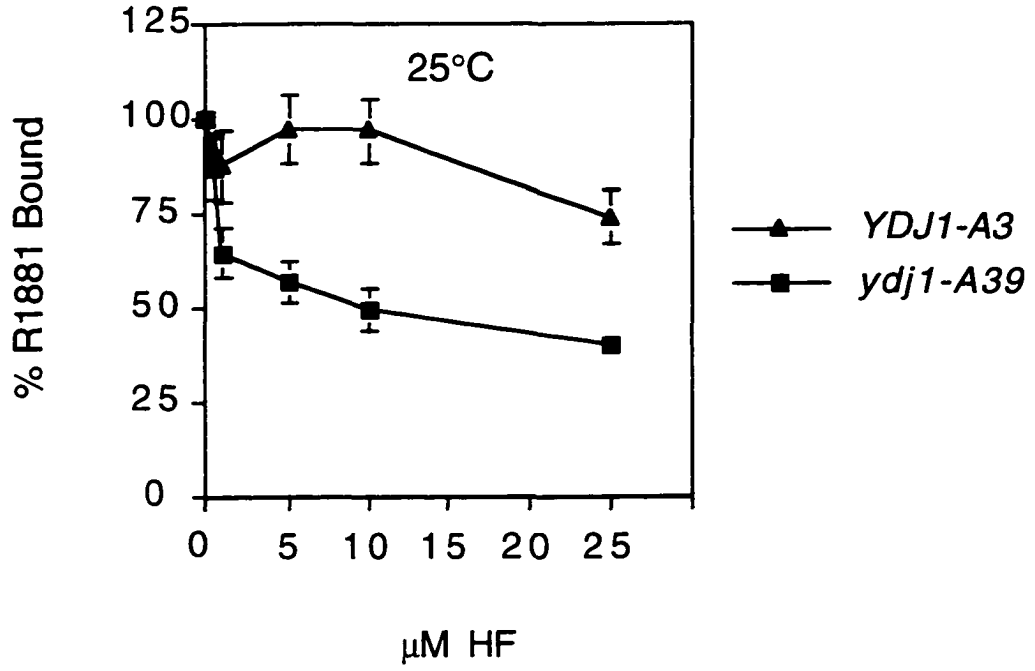


Figure 32. **HF competition assay on AR in wild type and *ydj 1-39* mutant yeast.** AR in wild type (*YDJ1*; closed triangles) and *ydj 1-39* (*ydj 1-A39*; closed squares) mutant cells were incubated at 25°C with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

(open diamonds). *ydj1-H34Q* mutant was able to partially complement the HF competition defect (Figure 31-open circles). In the presence of the *ydj1-H34Q* allele, 25 uM HF was able to compete R1881 binding to 50% of its original value, compared 80% for the wild type and about 30% for the other *ydj1* strains. Likewise, the *ydj1-39* or SIS1 mutant was unable to support wild type HF competition, (40 and 35% increase respectively) (Figure 32 and 31, respectively). When these experiments were performed with *ydj1-H34Q* and *SIS1* at 37°C a similar phenotype was observed (35-40% competition at 25 μM HF) (Figure 29B). This indicates that both the J domain is neccessary, but not sufficient for wild type HF competition. Likewise, both *ydj1-H34Q and SIS1* (open squares)expressed in trans were unable to complement the HF competition defect seen in the *Δydj1* strain (Figure 31). Also similar to the results for direct hormone binding, yeast containing the ydj1-G315D allele showed no alteration in HF competition when compared to that of the isogenic wild type strain. HF was only able to decrease R1881 binding to the AR to 70-80% of its original value in both the ydj1-G315D and isogenic wild type strains (Figure 33). These results indicate that some other domain of Ydj1 is required in addition to the J domain for wild type HF competition.

In order to determine whether these ligand binding defects manifests themselves in a downstream transactivation defect, lacZ reporter gene assays were performed on *ydj1* mutant yeast which were heterologously expressing AR. Expression of *ydj1-H34Q* was able to partially complement the transactivation defect seen in the *Δydj1* strain. When *Δydj1* yeast were

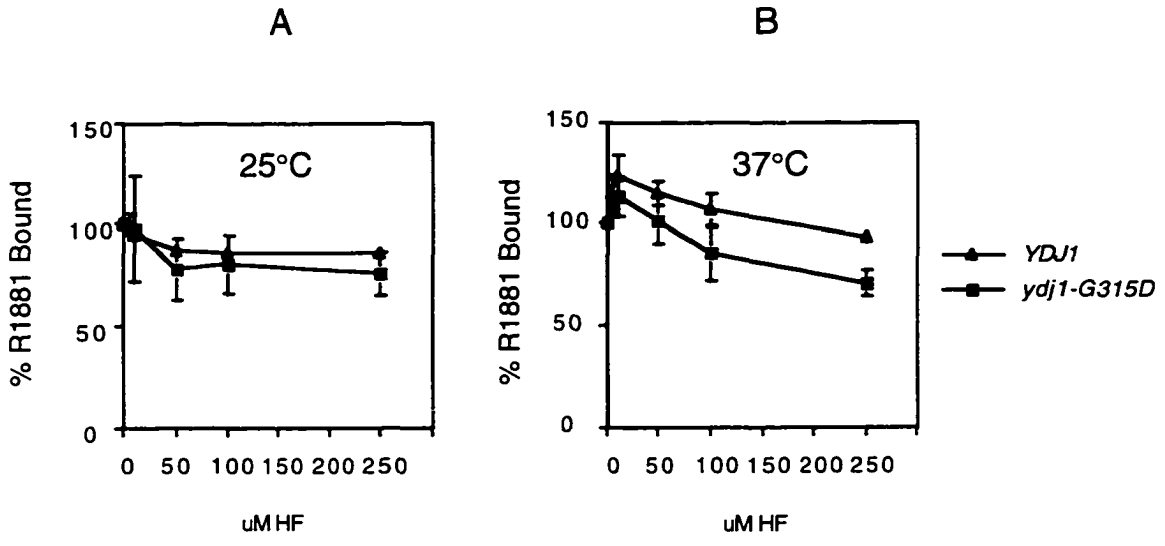


Figure 33. **HF competition assay on AR in wild type and *ydj1-G315D* mutant yeast.** AR in wild type (*YDJ1*; closed triangles) and *ydj1-G315D* (*ydj1-G315D*; closed squares) mutant cells were incubated at 25°C (A) and 37°C (B) with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

transformed with a plasmid containing the *ydj1-H34Q* allele, there was a 9-fold increase in DHT induced lacZ gene expression (Figure 34-lane 8). Likewise, overexpression of *SIS1* was able to partially complement the transactivation defect seen in the *Δydj1* strain. As seen in Figure 34, overexpression of *SIS1* (lane 6) led to a 4-fold increase in DHT induced lacZ gene expression. Expression of *ydj1-151* was only able to increase hormone dependent transactivation less than one fold over that of the *Δydj1* , even though it is essentially wild type for hormone binding This indicates that Ydj1p may play a dual role in AR activation, one role in hormone binding and one independent of hormone binding.

[3] Human Hdj2p, but not Hsj1 or Hdj1, Fully Complements the Ligand Binding Defect in the *Δydj1* Strain

Steroid hormone receptors such as the AR are not normally expressed in yeast, but are seen in higher eukaryotes including humans. In order to determine if higher eukaryote J proteins are capable of interacting with AR produced in the yeast, direct hormone binding and ligand competition assays were performed on wild type, *Δydj1* and *Δydj1* transformed with various human J homologues which were heterologously expressing the human AR. The human homologues tested were Hdj1. Hdj2 and Hsj1. Of these J homologues only Hdj2 (Type 1 J protein) contains the zinc finger domain and farnesylation signal as does Ydj1. Hdj1 and Hsj1 are Type 2 J proteins and contain only the J G/F domain in addition to the carboxy terminal domain (See for review

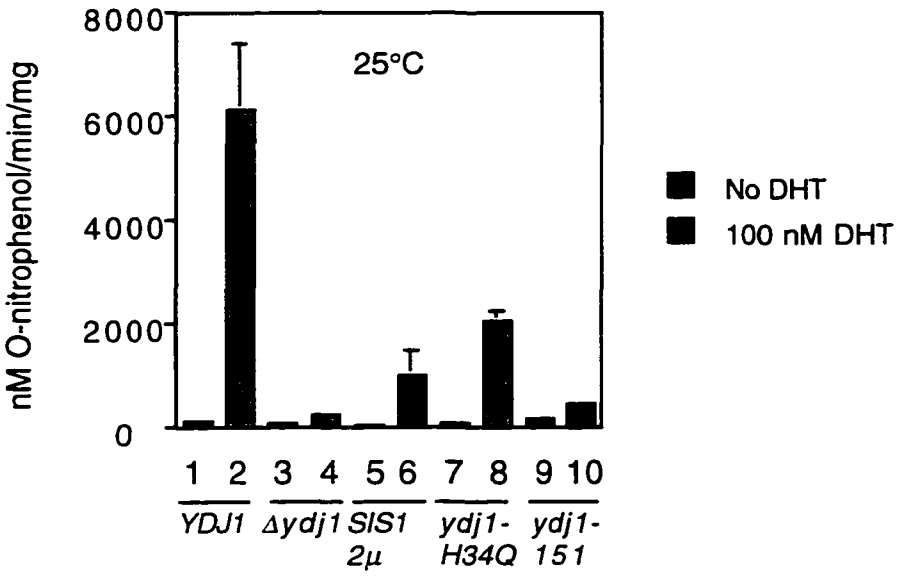


Figure 34. **Hormone dependent transactivation by AR in wild type and *ydj 1* mutant strains.** β-Galactosidase activity in wild type strain MYY290AR (*YDJ1*; lanes 1 and 2), *Δydj 1* strain MYY100AR (*Δydj 1*; lanes 3 and 4) mutant strains, *Δydj 1* cells transformed with *ydj 1-H34Q* strain AFY100H34Q (low copy number plasmid containing *ydj 1-H34Q* mutant allele) (*ydj 1-H34Q*, lanes 7 and 8), *Δydj 1* mutant yeast transformed with *SIS1* strain AFYSIS1 (*SIS1*, 2μ plasmid; lanes 5 and 6) (*SIS1* 2μ), and *Δydj 1* transformed with *ydj 1-151* containing plasmid strain AFY100-151(*ydj 1-151*; lanes 9 and 10) containing AR was measured in the presence (gray bars) or absence (black bars) of 100 nM DHT. Results are the mean of three independent experiments.

Cheetham and Caplan, 1997). All three human homologues were able to complement the Δydj1 growth defect at 25°C, but only Hdj2 was capable of complementing the temperature sensitive growth phenotype of the *Δydj1* strain at 37°C (Fig. 35). The results of the direct hormone binding assays are seen in Fig. 36. The only human J homologue that was able to complement the hormone binding defect seen in the *Δydj1* strain was Hdj2, the human Ydj1 homologue, even though receptor levels were similar in all strains as determined by Western blot analysis (Figure 37). Expression of Hdj2 was able to increase R1881 binding to the AR to levels comparable to the *Δydj1* co-expressing the wild type gene (Figure 36-open triangles). As seen in Fig. 35, expression of Hdj1p (open squares) or Hsj1p (crosses) was only able to increase R1881 slightly above that of the *Δydj1* strain.

In order to determine if any human homologues were capable of complementing the HF competition defect, ligand competition assays were performed on the wild type, *Δydj1* and *Δydj1* strains that had been transformed with human J homologues. Similarly, Hdj2 was the only human J homologue able to revert HF competition back to that of the wild type. As demonstrated in Fig. 38, in the Hdj2p (open triangles) expressing strain 25 uM HF was able to reduce R1881 binding to approximately 80% of the original value, compared to a reduction to 85-90% for the galYDJ1 (closed triangles) and 30-35% for the *Δydj1* strain (closed squares). In contrast, expression of Hdj1 (Figure 38-open squares) or Hsj1 (Figure 38-crosses) was unable to complement the HF competition defect in the *Δydj1* strain. In the Hdj1p and Hsj1p

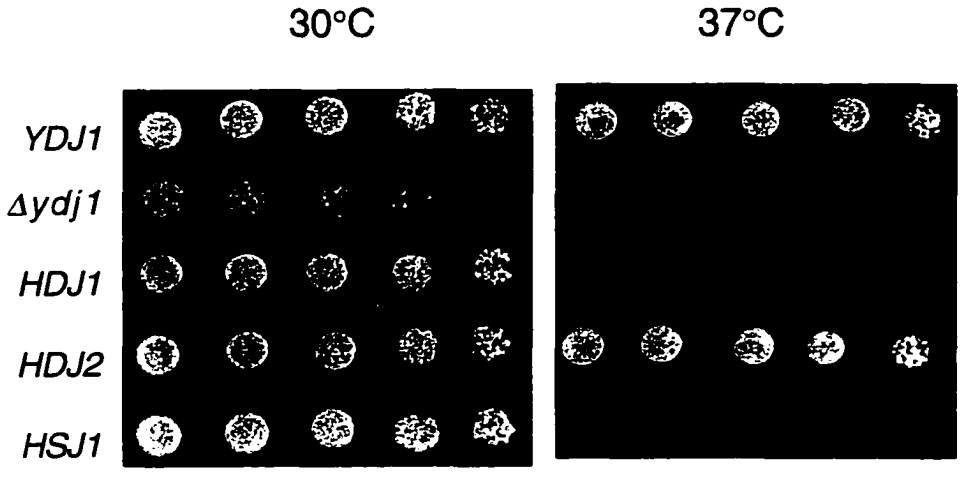


Figure 35. **Growth characteristics of the human J homologues in *Δydj1* mutant yeast**. Serial dilutions of yeast cells and growth at 30°C and 37°C in galactose. Wild type strain MYY290AR (*YDJ1*), *Δydj1* strain AFY100AR (*Δydj1*), *HDJ1* under the control of a galactose inducible promoter in *Δydj1* strain AFYHDJ1 (*HDJ1*), *HDJ2* under the control of a galactose inducible promoter in *Δydj1* strain AFYHDJ2 (*HDJ2*), *and HSJ1* under the control of a galactose inducible promoter in *Δydj1* strain AFYHSJ1 (*HSJ1*).

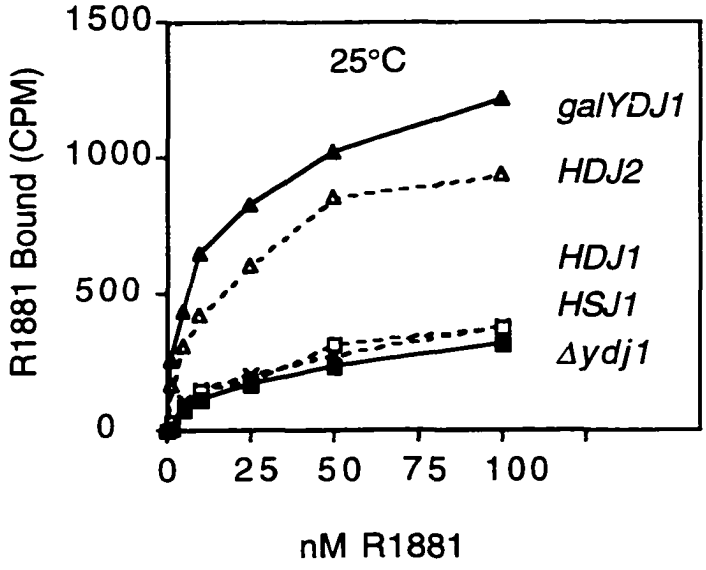


Figure 36. **Hormone binding to AR in *Δydj 1* mutant yeast transformed with Type 1 and 2 human J homologues.** Titration of 3H-R1881 in *Δydj 1* mutant yeast (*Δydj 1*; closed squares), *Δydj 1* wild type transformed with galYDJ1 strain AFYgalYDJ1 (*YDJ1* coding sequence under the control of a galactose inducible promoter) (galYDJ1; closed triangles), *Δydj 1* wild type transformed with *HDJ1* strain AFYHDJ1 (pRS315 containing *HDJ1* coding sequence under the control of a galactose inducible promoter) (*HDJ1*; open squares), *Δydj 1* wild type transformed with *HDJ2* strain AFYHDJ2 (pRS315 containing *HDJ2* coding sequence under the control of a galactose inducible promoter) (*HDJ2*; open triangles) and *Δydj 1* wild type transformed with *HSJ1* strain AFYHSJ1 (pRS315 containing *HSJ1* coding sequence under the control of a galactose inducible promoter) (*HSJ1*; solid crosses) mutant yeast cells heterologously expressing AR were tested for R1881 binding at 25°C. Results are the mean of three independent experiments.

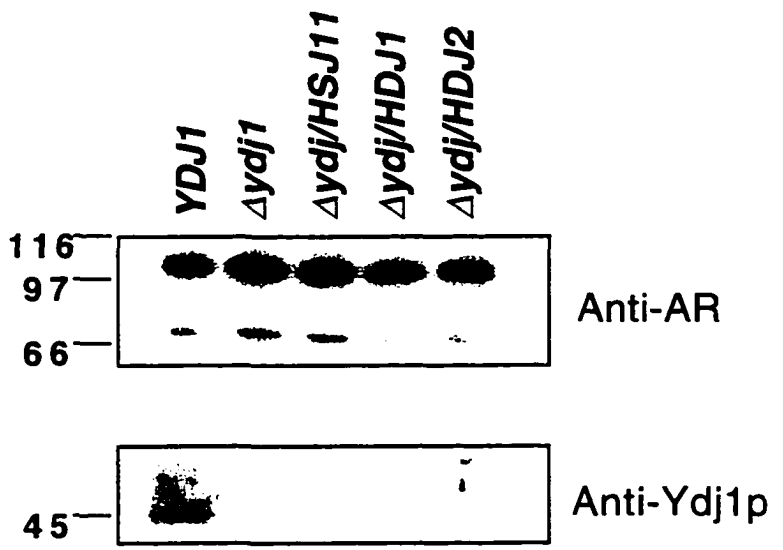


Figure 37. **Western blot analysis of AR with human J homologues.** (top panel; arrow denotes location of AR and star denotes breakdown product or AR), Ydj 1 (second panel; arrow denotes location of Ydj 1). Analysis was performed on whole cell extracts of *YDJ1* strain MYY290AR (*YDJ1*; lane 1), *Δydj 1* strain AFY100AR (*Δydj 1*; lane 2), *HDJ1* in *Δydj 1* strain AFYHDJ1 (*HDJ1*; lane 3), *HDJ2* in *Δydj 1* strain AFYHDJ2 (*HDJ2*, lane 4) and *HSJ1* in *Δydj 1* strain AFYHSJ1 (*HSJ1*, lane 5) yeast expressing AR. Molecular weight standards are shown in kDa.

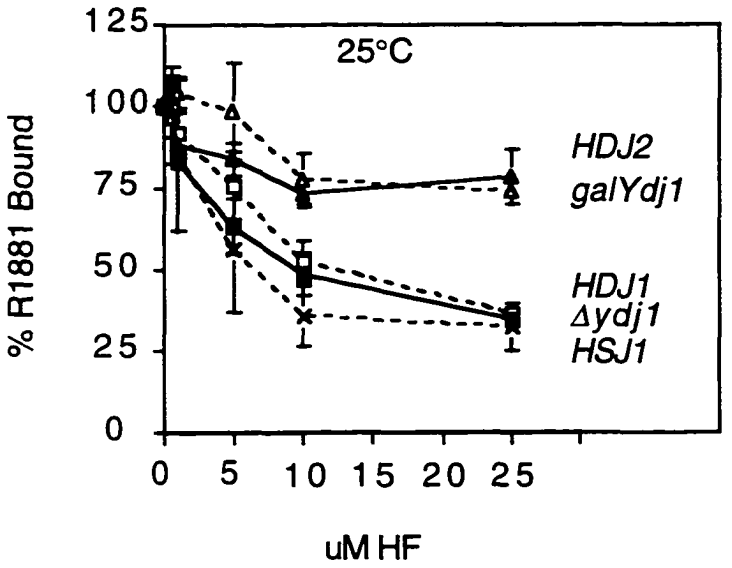


Figure 38. **HF competition assay on AR in *Δydj 1* mutant yeast transformed with Type 1 and 2 human J homologues.** Heterologously expressed AR in *Δydj 1* mutant yeast strain AFY100AR (*Δydj 1*; closed squares), *Δydj 1* wild type transformed with galYDJ1 strain AFYgalYDJ1 (*YDJ1* coding sequence under the control of a galactose inducible promoter) (galYDJ1; closed triangles), *Δydj 1* yeast transformed with *HDJ1* strain AFYHDJ1 (pRS315 containing *HDJ1* coding sequence under the control of a galactose inducible promoter) (*HDJ1*; open squares), *Δydj 1* wild type transformed with *HDJ2* strain AFYHDJ2 (pRS315 containing *HDJ2* coding sequence under the control of a galactose inducible promoter) (*HDJ2*; open triangles) and *Δydj 1* wild type transformed with *HSJ1* strain AFYHSJ1(pRS315 containing *HSJ1* coding sequence under the control of a galactose inducible promoter) (*HSJ1*; solid crosses) mutant yeast cells heterologously expressing AR were incubated at 25°C with increasing amounts of μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

expressing strains, 25 uM HF reduced R1881 binding to approximately 30-40% of its original value, compared to about 35% for the mutant and 85-90% for the wild type strains.

In order to determine whether this hormone binding manifests itself in a downstream transactivation defect, lacZ reporter gene assays were performed on Δydj1 yeast containing human homologues of Ydj1 which were heterologously expressing AR. As with both hormone binding and HF competition, expression of *HDJ2* was able to fully complement the decrease seen in hormone induced lacZ gene expression seen in the *Δydj1* yeast (Figure 39). As seen in Figure 39, DHT induced lacZ gene expression was virtually identical in both wild type and *Δydj1* yeast transformed with *HDJ2*. Expression of *HDJ1* or *HSJ1* were unable to complement the transactivation defect seen in the *Δydj1* strain. As seen in Figure 39, DHT induced lacZ gene expression was virtually identical in both *Δydj1* and *Δydj1* yeast transformed with either *HDJ1* (lane 6)or *HSJ1* (lane 10).

[4] Hormone Binding to the ER is Decreased in the Absence of Ydj1p

Previous studies suggest that ER signaling is defective in the absence of functional Ydj1p (Kimura et al., 1996). In this study, there was a deregulation of ER activation in the *ydj1-G315D* mutant, even in the absence of hormone the receptor was able to transactivate a target gene. In order to confirm this transactivation defect, lacZ reporter gene assays were performed on wild type

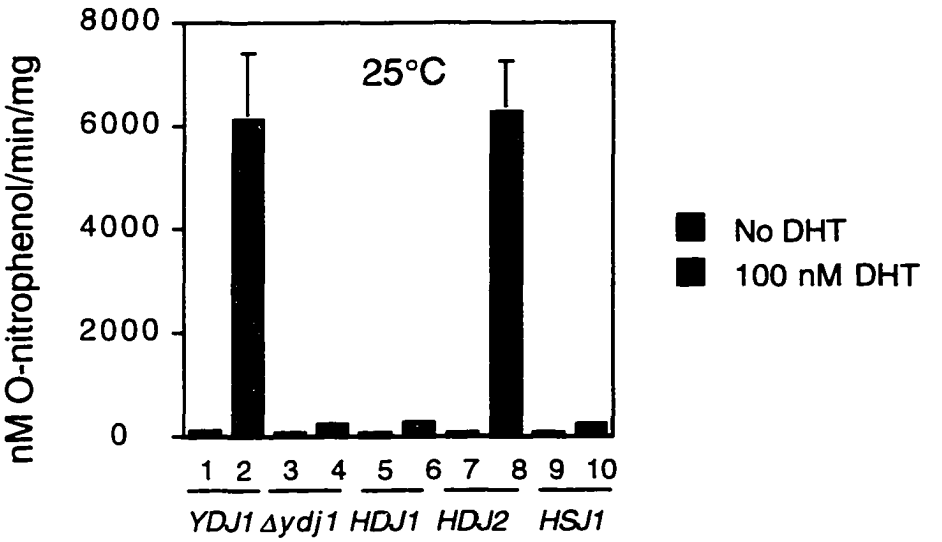


Figure 39. **Hormone dependent transactivation by AR in *Δydj 1* mutant yeast transformed with Type 1 and 2 human J homologues.** ß-Galactosidase activity in wild type strain MYY290AR (*YDJ1*; lanes 1 and 2), *Δydj 1* strain AFY100AR (*Δydj 1*; lanes 3 and 4), *Δydj 1* wild type transformed with *HDJ1* strain AFYHDJ1 (pRS315 containing *HDJ1* coding sequence under the control of a galactose inducible promoter) (*HDJ1*; lanes 5 and 6), *Δydj 1* wild type transformed with *HDJ2* strain AFYHDJ2 (pRS315 containing *HDJ2* coding sequence under the control of a galactose inducible promoter) (*HDJ2*; lanes 7 and 8) and *Δydj 1* wild type transformed with *HSJ1* strain AFYHSJ1 (pRS315 containing *HSJ1* coding sequence under the control of a galactose inducible promoter) (*HSJ1*; lanes 9 and 10) mutant yeast cells heterologously expressing AR was measured at 25°C in the presence (gray bars) or absence (black bars) of 100 nM DHT. Results are the mean of three independent experiments.

and *Δydj1* mutant yeast that heterologously expressed the ER in addition to containing a lacZ reporter gene under the control of an estrogen response element. In contrast to previous studies with *ydj1-G315D*, *Δydj1* yeast were significantly decreased in their ability to transactivate upon hormone binding when compared to the wild type strain (Figure 40). There was essentially no induction of lacZ gene expression upon incubation with E2 in the Δydj1 yeast strain.

In order to determine whether this transactivation defect seen in the absence of functional Ydj1p was in part due to an alteration in hormone binding, direct hormone binding and 4-OHT competition assays were performed on wild type and *Δydj1* yeast which were heterologously expressing the human ER. As shown in Fig. 41A, DES binding to the ER in the *Δydj1* strain was reduced by approximately 4-fold compared to the wild type strain at 100 nM DES, although both wild type and mutant strains contained similar amounts of ER (Figure 41B). This indicates that Ydj1 is required for wild type hormone binding to the ER. Interestingly, while 4-OHT could compete for hormone binding in the wild type strain, it had no effect (neither competition nor potentiation) on DES binding to the ER in the *Δydj1* strain (Figure 41A). Likewise as expected, the ER hormone binding defect was fully complemented when the wild type *YDJ1* gene was added back to the *Δydj1* strain (Figure 41A-lanes 3 and 4). This indicates that Ydj1p is required for wild type hormone binding to the ER.

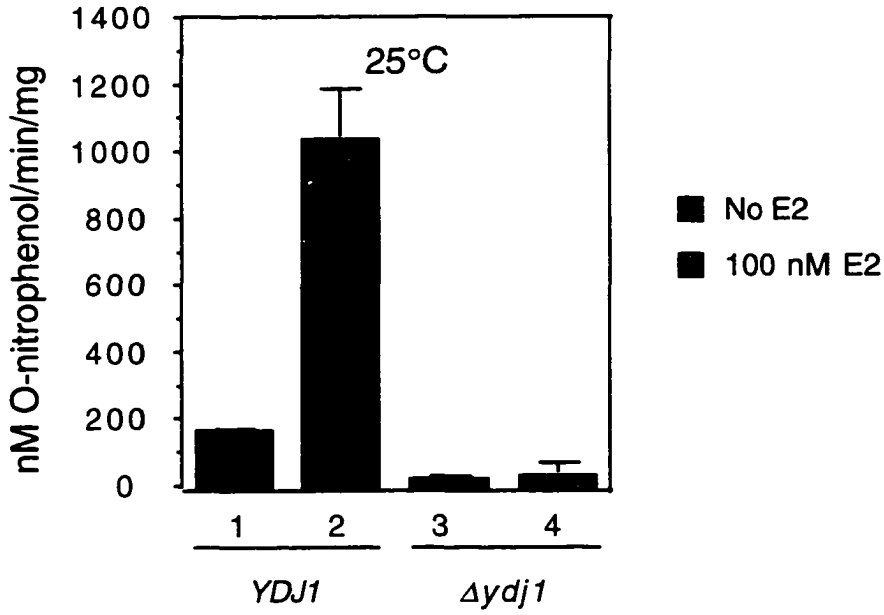


Figure 40. **Hormone dependent transactivation by ER in wild type and *Δydj1* mutant yeast**. ß-Galactosidase activity in wild type strain W3031ER(*YDJ1*; lanes 1 and 2), *Δydj1* strain ACY95ER(*Δydj1*; lanes 3 and 4) mutant yeast cells heterologously expressing ER was measured at 25°C in the presence (gray bars) or absence (black bars) of 100 nM E2. Results are the mean of three independent experiments

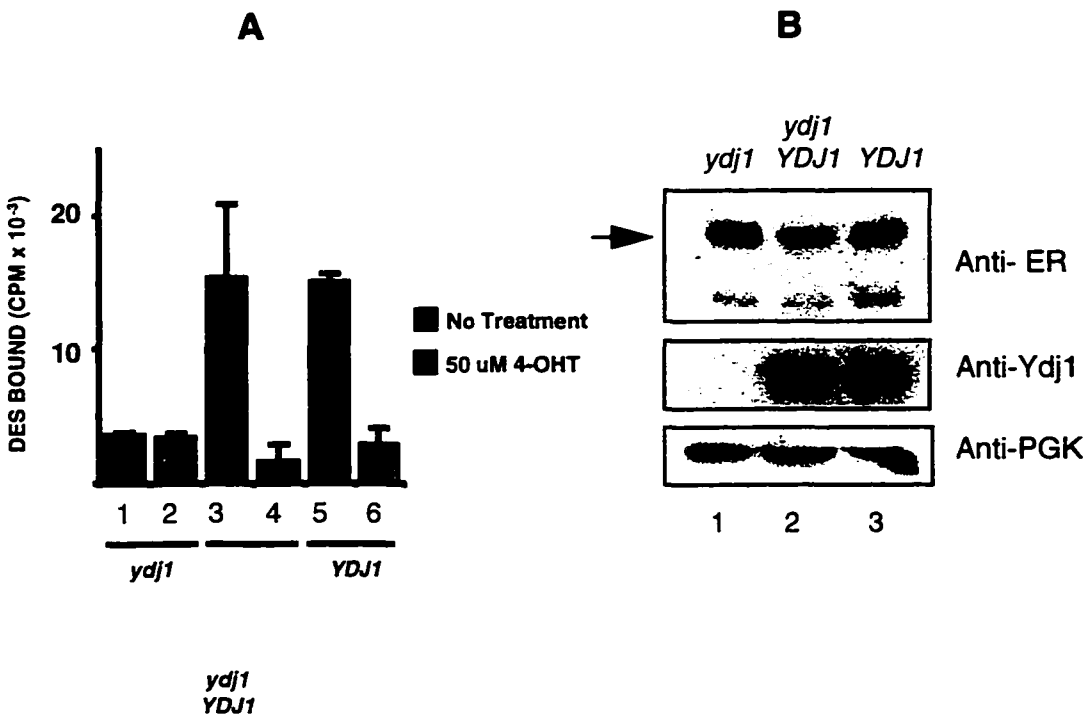


Figure 41. **4-OHT competition assays on *YDJ1* and *Δydj1* yeast strains.** (A) Wild type strain W3031ER (*YDJ1*; lanes 5 and 6), *ydj1* null strain ACY95ER (*Δydj1*; lanes 1 and 2) and *ydj1* null covered by wild type *YDJ1* on a plasmid strain ACY95pAV7 (*Δydj1 YDJ1*) yeast heterologously expressing ER were incubated with (+) or without (-) 50 μM 4-OHT in the presence of 100 nM [^{3}H] DES. Results are expressed as DES bound (cpm x 10^{-2}). Results are the mean of three independent experiments. (B) Western blot analysis or ER (top panel; arrow denotes location of ER and star denotes breakdown product or ER) and Ydj1 (middle panel; arrow denotes location of Ydj1). Analysis was performed on whole cell extracts of *ydj1* null strain ACY95ER (*Δydj1*; lane 1), *ydj1* null covered by a plasmid containing wild type *YDJ1* on a plasmid strain ACY95pAV7 (*Δydj1 YDJ1*; lane 2) and wild type strain W3031ER (WT; lane 3) yeast expressing ER. 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).

Discussion

In this study it was shown that mutation or deletion of Ydj1p affects the ability of both the AR and ER to bind ligand and subsequently transactivate target genes. First, deletion of Ydj1p leads to a decrease in the ability of AR and ER to bind hormone (Figure 24 and 41). In the *Δydj1* strain, AR was unable to bind the synthetic androgen R1881 to the same extent as in the isogenic wild type strain. Likewise, ER was unable to bind DES at wild type levels in the *Δydj1* strain. Second, the absence of Ydj1p affected the ability of HF to compete for hormone binding to the AR and the ability of the drug 4-OHT to compete for DES binding to the ER. In the *Δydj1* mutant strain, HF acted as a more potent competitor of R1881 binding to the AR (Figure 26), whereas, in the ER expressing strain, 4-OHT was unable to compete for DES binding (Figure 41A). In addition these hormone binding defects seen with the AR could be partially or completely complemented by various Ydj1 mutants and yeast or human homologues. Both *ydj1-H34Q* (J domain mutant) and the yeast homologue *SIS1* (does not contain a zinc finger domain or farnesylation signal) were able to partially complement the hormone binding defect (Figure 27). Likewise, the human Ydj1 homologue, Hdj2 was able to fully complement both the growth (Figure 35) and hormone binding defects (Figure 36 and 38). This suggests that Ydj1 plays an important role in hormone binding of both the AR and ER and since the J domain is required for this function it may be suggested that Ydj1 is acting via Hsp70.

Ydj1 plays an important role in hormone dependent signaling of the AR (Caplan et al., 1995) via its HBD, suggesting that the defect is a hormone binding defect. Dittmar et al. recently demonstrated using an *in vitro* reconstitution system that GR is unable to bind hormone upon depletion of hsp40 (J protein) and this hormone binding defect can be reversed upon the addition of Ydj1 protein to the extract (1998). Likewise, the results presented here are consistent with a role for Ydj1 in maintaining the AR in the hormone binding state *in vivo*, since in the absence of Ydj1 AR has a decreased ability to bind R1881 (Figure 24). Likewise, HF acts as a more potent competitor of R1881 binding to the AR in the absence of Ydj1p, as it did in the absence of functional Hsp90 (Figure 26). Both of these results suggest that Ydj1 is required for AR hormone binding *in vivo*.

Ydj1 can be broken up into three major domains; 1) J G/F domain which is responsible for increasing the normal low level of ATPase of Hsp70 (Liberek et. al., 1991), 2) zinc finger domain which has chaperoning function of its own and is able to prevent the aggregation of proteins (Lu and Cyr, 1997; Szabo et. al., 1995), and 3) carboxy-terminal domain which also has chaperoning function of its own and is able to prevent the aggregation of proteins (Lu and Cyr, 1997). Ydj1 may be functioning via Hsp70 in AR hormone binding and transactivation, since upon depletion of Hsp70 the GR is unable to bind hormone *in vitro*. The results presented here are consistent with the idea that Ydj1 is acting at least in part via Hsp70 in AR signaling, since *ydj1-H34Q* which lacks a functional J domain is able to partially complement both the hormone

binding (Figure 27 and 29) and ligand competition (Figure 29 and 31) phenotype seen in the *Δydj1* strain. This suggests that the J domain is neccessary, but not sufficient for wild type hormone binding. These results also suggests that the some other domain or combination of domains is required in addition to the J domain for wild type hormone binding. Interestingly, AR in the carboxy-terminal mutant *ydj1-G315D* demonstrated no alteration in hormone binding (Figure 30) or ligand competition (Figure 33), suggesting that this region is not essential for hormone binding. Similar to the results shown for the *ydj1-H34Q* mutant, overexpression of *SIS1* in the *Δydj1* strain was able to partially complement the hormone binding defect (Figure 27 and 29), but was unable to complement the ligand competition defect (Figure 29 and 31). Taken together these data indirectly suggest that either the zinc finger domain and/or farnesylation is also required for hormone binding to the AR, since overexpression of the J/GF domain of SIS1 is able to only partially complement the ligand binding defects. However, this may be in part due to the fact that the J/GF domain of SIS1 is not identical to that of Ydj1. Interestingly, expression of both domain the J/GF and zinc finger domain/farnesylation in trans was not able to further complement the hormone binding (Figure 27) or ligand competition defect (Figure 31). So one could speculate that these regions of Ydj1 must be together in three-dimensional space in order to coordinate their activities. Likewise, hormone dependent transactivation was only partially complemented by expression of both *ydj1-H34Q* and *SIS1* 2μ. None of these defects could be accounted for by

decrease in level or stability of the AR, since receptor levels were similar as determined by Western blot analysis. Since previous studies have demonstrated that the J domain functions in association with Hsp70, one would speculate that Ydj1 is acting at least partially via Hsp70 in hormone binding to the AR. Although, it can not be discounted that the J domain may have additional functions independent of the Hsp70 reaction cycle.

In its native environment, the human AR does not normally interact with yeast molecular chaperones, but rather it would associate with human homologues of these same chaperones. Since the chaperone machinery is conserved functionally from yeast to higher eukaryotes (Chang and Lindqusit, 1994; Stancato et. al., 1996), it would be expected that the appropriate human J homologue may be able to complement the hormone binding defect seen in the *Δydj1* strain. Based on the previously described analysis of yeast Ydj1 mutants and homologue earlier in this chapter, one might postulate that a Type 1 J protein would be able to complement the AR signaling defect in the *Δydj1* strain. Consistent with this notion, neither of the Type 2 J proteins, Hdj1 and Hsj1, tested were able to complement either the hormone binding (Figure 36) or ligand competition (Figure 38) defect seen in the *Δydj1* strain. The only human J homologue that was able to complement the binding and ligand competition defect seen in the mutant was the Ydj1 homologue, Hdj2 (Figure 36 and 38). Hdj2 was able to fully complement hormone binding defects (Figure 36 and 38) as well as the temperature sensitive growth defect of the *Δydj1* strain (Figure 35). Likewise, expression of Hdj2 was also able to fully

complement the transactivation defect seen in the *Δydj1* strain (Figure 39). This is consistent with the idea that both the J/GF domain in combination with either the zinc finger domain and/or farnesylation are important for hormone binding to the AR. Thus it is likely that J proteins are functioning at least partially via Hsp70, since the J domain aids in Hsp70 function. This data also suggests that in its native environment the AR may require the action of Hdj2 for proper steroid receptor function.

It has also been suggested that Ydj1 plays a vital role in ER signaling (Caplan et. al., 1995; Kimura et. al., 1995). This previous study demonstrated a deregulation of hormone dependent transactivation by the ER in a *ydj1* mutant strain. In the *ydj1-G315D* strain, there were increased levels of hormone independent activity. In contrast, results presented here have not shown this same deregulation of hormone dependent transactivation. The ER was decreased in its ability to transactivate upon hormone binding, in the absence of Ydj1 (Figure 40). Likewise, this transactivation defect was at least in part caused by a decrease in the ability of the ER to bind hormone. Similar to AR, there was also a concurrent alteration in the ability of 4-OHT to compete for hormone binding, again suggesting that ligand binding is defective in the absence of Ydj1p. In the *Δydj1* strain, 4-OHT was neither able to compete (as in wild type) nor potentiate (as in the *hsp82* mutant strains) hormone binding to the ER (Figure 41A), suggesting that Ydj1 exerts its affects at a different stage in the maturation of the ER to a hormone binding competent state than that of Hsp90.

The results presented here suggest that Ydj1 or perhaps more correctly its mammalian counterpart Hdj2 is required for both AR and ER signaling. It has been previously demonstrated that the conserved J domain is essential for Ydj1's function in the Hsp70 reaction cycle. So it is likely that Ydj1 or its mammalian counterparts exert their affects via Hsp70 in steroid receptor signaling.

Experimental Methods

Materials

DHT, DES and 4-OHT were obtained from Sigma Chemical Company. Cold R1881 was obtained from NEN. The above compounds were solubilized in ethanol and stored at -20°C. Antisera to ER was a kind gift of Dr. G. Greene. Antisera to AR and Ydj1p was previously described (Fang et. al., 1996; Caplan et. al., 1995). Antisera to yeast phosphoglycerate kinase was a kind gift of Dr. P. Lazarow. ^{3}H DES and ^{3}H R1881 were purchased from American Radiochemical Company and Amersham, respectively. PCY7 (*ydj1-G315D*) and YPH488 (isogenic wild type) were a gift from Dr. Susan Lindquist. Yeast strains A3 (isogenic wild type) and A39(*ydj1* mutant) were a gift from Dr. Frank Boschelli.

Yeast Strains and Growth Conditions

Yeast cells were cultured in selective media (0.67% yeast nitrogen base, 2% glucose or 2% galactose plus the appropriate amino acids) using standard procedures. The *YDJ1* and *Δydj1* parental strains used for the AR studies were MYY290 (MAT a, leu2, his3, ura3) and MYY405 (MAT a, leu2, his3), respectively (Atencio and Yaffe, 1992). MYY405 was plated on FOA to deselect for the URA3 marker and this strain AFY100 was used as the parental strain for the remainder of this study. Plasmid transformations were performed by the LiAc procedure as described previously (Geitz et al., 1995). Plasmids used in this study were pG1-hAR (human AR); Purvis et al., 1991), pPGKareLacZ (lacZ reporter gene under control of androgen response elements, URA3; Purvis et al., 1991), p2HGPDER/CYC (human ER, 2μ, HIS3; Kimura et al., 1995), pUCΔSS-ERE (lacZ reporter gene under control of estrogen response element, URA3; Picard et. al., 1990), *ydj1-H34Q* (Tsai and Douglas, 1996), *HDJ1* , *HDJ2* , *HSJ1* , *SIS1*(2μ, URA3;) , *ydj1-151* (CEN/ARS, LEU2; Caplan et al., 1995), pGALYDJ1(GAL 1 promoter, URA3) , pARH and pARU (Caplan et al., 1995; Fang et. al. 1996 and Chapter 1 of this thesis) and pAV7(*YDJ1*, CEN/ARS/LEU2; Caplan et al., 1995). AFY100 (MATa, *Δydj1*, leu2, his3, ura3) transformed with plasmids containing *ydj1-151*, *YDJ1*, *ydj1-H34Q*, *HDJ2* or *HSJ1* was subsequently transformed with pARH and pPGKarelacZC. AFY100 (MATa, *Δydj1*, leu2, his3, ura3) transformed with plasmids containing pgalYDJ1 and SIS1 were subsequently transformed with pARH and pRS315arelacZ. AFY100 (MATa, *Δydj1*, leu2, his3, ura3) previously

transformed with plasmids containing HDJ1 was subsequently transformed with pARU and pRS315arelacZ. PCY7 (*ydj1-G315D*) (Kimura et. al., 1995) and YPH488 (isogenic wild type) (Kimura et. al., 1995) were deselected for pGN795(GR, TRP1; Schena and Yamamoto) and subsequently transformed with pG1-hAR. Likewise, yeast strains A3 and A39 were transformed with pARU.

Plasmid Subcloning

The promoter and open reading frame for *ydj1-H34Q* was subcloned into the vector pRS316(CEN/ARS, LEU2). YQPD was digested with Eag1 and Xho1 and the fragment was gel purified and ligated into similarly digested pRS315. The open reading frame for *HDJ2* was subcloned into the vector pRS315gal (GAL1 promoter, CEN/ARS, LEU2). HDJ2 was digested with BamH1 and Eag1 and the appropriate fragment was gel purified and ligated into similarly digested pRS315gal. Likewise, HSJ1 was digested with BamH1 and the appropriate fragment was gel purified and ligated into similarly digested pRS315gal(GAL1 promoter, CEN/ARS, LEU2). The arelacZ from pPGKarelacZ was subcloned into a LEU2 vector. pPGKarelacZ was digested with HindIII and Sal1 and the appropriate fragment was ligated into similarly cut vector and was termed pRS315arelacZ.

ß-Galactosidase Activity Assay

Yeast cells were grown to early log phase (OD^{600}= 0.2) and preincubated at either 25°C or 37°C for one hour prior to the addition of either DHT or E2. These cells were then incubated for an additional 2 hours at the same temperature prior to harvesting and the preparation of extracts as previously described (Caplan et. al., 1995) ß-galactosidase activity assays were performed as previously described (Caplan et. al., 1995).

Ligand Binding Assays

Yeast cells were grown in selective media containing 2% glucose or 2% galactose to early log phase (OD^{600}= 0.2) and 1 ml aliquots were subsequently incubated at either 25°C or 37°C for 30 minutes. Following this preincubation, cells were incubated with either ^{3}H R1881 for the AR or ^{3}H DES for the ER for an additional 1.5 hours at the same temperature. The cells were then washed 3 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 R1881 for the AR or DES for the ER from the samples incubated in the absence of cold R1881 or DES.

Ligand competition assays were also performed with yeast cells which were grown to early log phase (OD^{600}= 0.2) as described above. Following a 30 minute preincubation at either 25°C or 37°C, cells were incubated with 100 nM

^{3}H R1881 for the AR and 100 nM ^{3}H DES for the ER in the presence or absence of varying concentrations of HF for the AR and 4-OHT for the ER for an additional 1.5 hours at the same temperature. The cells were then washed 3 times with 1 ml of water each and counted in 5 ml of scintillation fluid.

Western Blot Analysis

The levels of AR, ER and Ydj1 were assayed by Western blot analysis using either anti-AR, anti-ER or anti-Ydj1 specific antibodies. Yeast lysates were prepared as previously described (Caplan et. al., 1995). 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 and blocked overnight at room temperature with TTBS containing 5% non-fat dry milk. Filters were subsequently incubated with antibodies specific for the AR, ER or Ydj1 (antibodies were diluted in antibody dilution buffer, 1x PBS, 3% bovine serum albumin, 0.05% Tween 20 and 0.1% thimerosal (1:1000 for anti-Ydj1 and anti-AR and 1:2000 for anti-ER) for either 1 hour (anti-Ydj1 or anti-AR) or 4 hours (anti-ER). Filters were washed three times for 10 minutes 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-AR and anti-Ydj1) for 1 hour and subsequently washed three times for 10 minutes each in TTBS. Filters were treated with the chemiluminescence reagent (Pierce) and exposed to X-ray

film. An identical filter was probed with anti-PGK (1:300,000 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-Ydj1, anti-AR and anti-ER.

CHAPTER IV

CHARACTERIZATION OF THE ROLE OF CDC37 IN ANDROGEN, ESTROGEN AND GLUCOCORTICOID RECEPTOR ACTION

Introduction

The mammalian p50 protein is known to interact with Hsp90 in the v-src activation complex (Whitelaw et al., 1991, Brugge, 1986). In addition, both p50 and Hsp90 were also identified as vital components of the signal transduction pathway originating from the Drosophila Sevenless receptor which is involved in eye development (Cutforth and Rubin, 1994). Recently the essential yeast cell division cycle gene CDC37 was identified as an orthologue of mammalian p50 (Reed, 1980). Both mammalian p50 and yeast CDC37 are involved in the stabilization of cyclin dependent kinases (Gerber et. al., 1995; Stepanova et. al., 1996; Dai et. al., 1996) Yeast CDC37 plays an important role in the stabilization of Cdc28 (Gerber et. al., 1995). In the case of the mammalian p50, it is involved in the stabilization of CDK4 (Stepanova et. al., 1996), suggesting a conservation of function from higher to lower eukaryotes. Likewise, recent genetic studies with yeast demonstrate that mutations in CDC37 cause a reduction in the v-src activity that is normally lethal in the yeast (Florio et. al., 1994; Brugge et. al., 1987; Jove and Hanafusa, 1987; Kornbluth et. al., 1987; Boschelli et. al., 1993; Xu and Lindquist, 1993), giving further evidence for conservation, supporting the involvement of Cdc37 in kinase action. However to date, p50/CDC37 has not been seen in physical association with steroid hormone aporeceptor complex (Nair et al, 1997; Whitelaw et al., 1991; Stancato et al., 1993), suggesting that p50/CDC37 might be specific for protein kinase signal transduction pathways. However, the events that lead to formation of the Hsp90 complex with v-src and steroid

receptors are indeed quite similar, suggesting that p50/CDC37 might play a role in receptor activation. In order to address the question of the role of p50/CDC37 in steroid receptor activation, it was tested as to whether AR, ER and GR signaling was affected by a mutation in the yeast CDC37.

Results

A temperature sensitive *cdc37* mutant strain was used to study hormone binding and subsequent hormone dependent transactivation by heterologously expressed AR, ER and GR, in order to determine whether Cdc37p is required for steroid hormone receptor function. The mutant used in this study is termed *cdc37-34* and was previously described as being a suppressor of v-src lethality in *Saccharomyces cerevisea* (Dey et al., 1996). This mutant grows slowly at 25°C (the permissive temperature) and is inviable at 37°C (the restrictive temperature) (Figure 42). Sequence analysis of this allele revealed a single base pair substitution (C to T) at nucleotide 41 in the open reading frame (sequence analysis performed by Dr. Robert Donnelly, UNJ). This point mutation resulted in the change converting residue 14 from a serine to a leucine. This residue is one of two serines phosphorylated by casein kinase II *in vivo* and if both of these residues are converted to alanine Cdc37p has reduced function (personal communication from Dr. Glover). Both of these phosphorylated serines are in the highly conserved region of Cdc37p which is residue 1-38. This region is 50% identical and 70% conserved from yeast to human (Figure 43), suggesting a conservation of function.

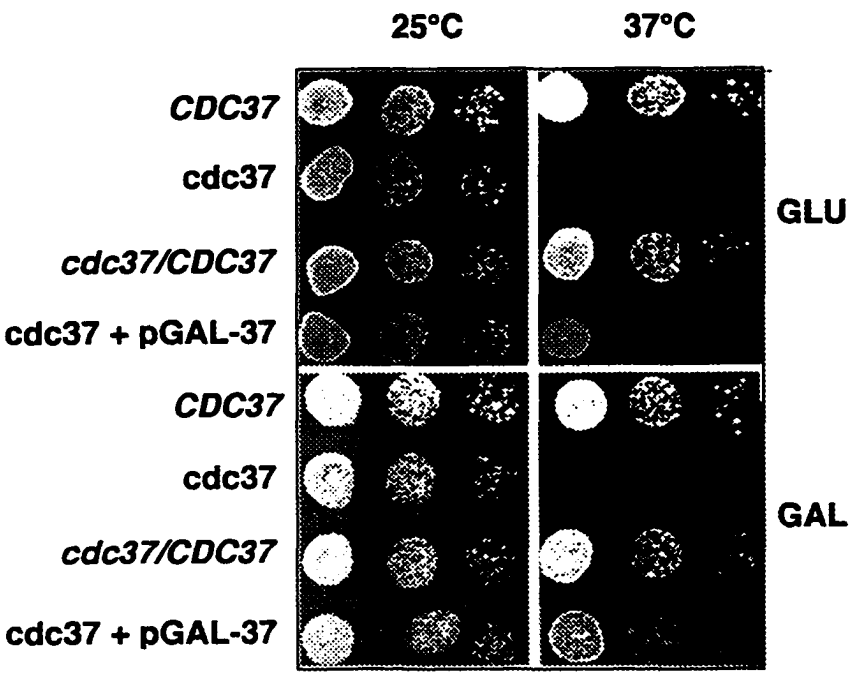


Figure 42. **Growth characteristics of the *cdc37-34* mutant strain.** Serial dilutions of yeast cells and growth at 25°C and 37°C. Wild type strain AFY17 (*CDC37*), *cdc37* strain AFY14 (*cdc37-34*), *cdc37-34* with pRSS2 strain AFY16 (low copy number plasmid with *CDC37* (*cdc37/CDC37*), *cdc37-34* with multicopy plasmid containing *CDC37* under galactose promoter control strain AFY18 (*cdc37*/pGAL-37).

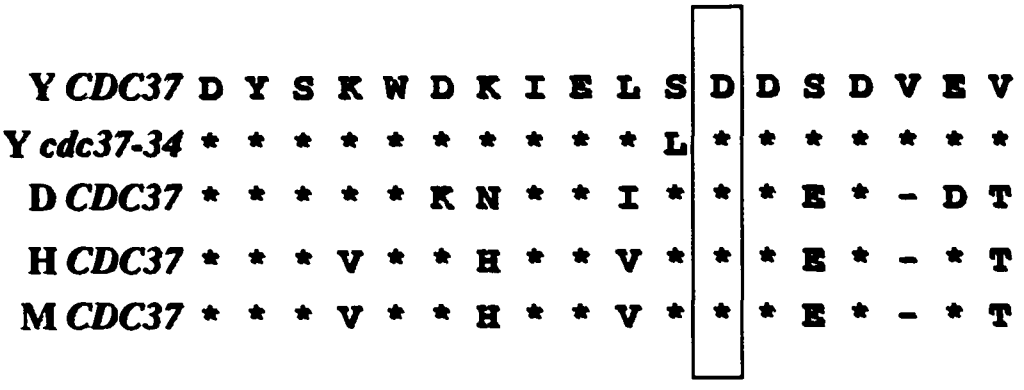


Figure 43. **Cdc37 protein sequences**. Comparison of the N-terminal region of p50/Cdc37 proteins in single letter amino acid code from *S. cerevisiae* (Y), *Drosophila melanogaster* (D), human (H), and mouse (M). Conserved residues are denoted by a star. The position of the S14L mutation in *cdc37-34* is shown by an arrow.

[1] Cdc37p Functions in the Activation of the AR

It has been suggested that p50/Cdc37 only functions in association with Hsp90 in the regulation of protein kinases such as v-src, Sevenless and cyclin dependent kinases (CDK4 and Cdc28) (Brugge et. al., 1987; Cutworth and Rubin, 1996; Stepanova et. al., 1996; Gerber et. al., 1995; Whitelaw et. al., 1991), since it is not seen in stable physical association with Hsp90 in the aporeceptor complex. In order to answer the question is Cdc37p required for receptor activation, the *cdc37-34* mutant and an isogenic wild type were assayed for DHT dependent lacZ expression driven by heterologously expressed AR at both the permissive (25°C) and non-permissive (37°C) temperatures. As seen in Figure 44, when assays were performed with the wild type strain using 100 nM DHT there was a 22 fold induction of lacZ activity over the background at 25°C (Figure 44A-lane 2) and a 79 fold induction for the same at 37°C (Figure 44B-lane2). In contrast, when these assays were performed on the *cdc37-34* mutant strain there was only a 4 to 5 fold induction with DHT at 25°C (Figure 44A-lane 4) and less than a 1 fold induction at 37°C (Figure 44B-lane 4). Comparing the wild type to the *cdc37-34* mutant, there was a 5 fold decrease in DHT induced activity at 25 °C and almost 80 fold decrease at 37°C. This transactivation defect was not caused by decreased stability or expression of the AR, since AR levels as determined by Western blot are similar in both the *cdc37-34* mutant and wild type extracts (Figure 45). Transactivation by the AR is therefore defective in the *cdc37-34* mutant strain at both 25° and 37°C, however, the phenotype is much more dramatic at 37°C.

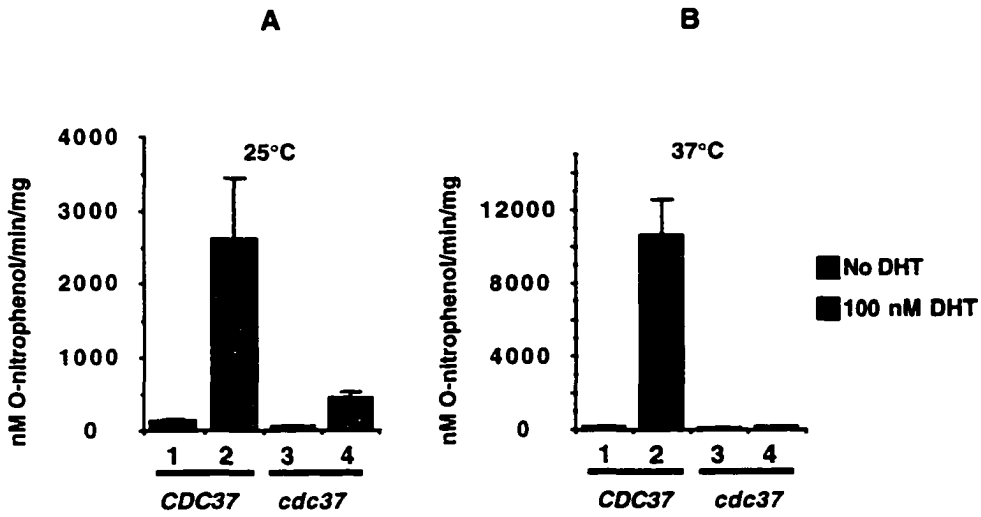


Figure 44. **Hormone dependent transactivation by AR is defective in a *cdc37* mutant strain.** (A) ß-Galactosidase activity in wild type strain AFY17 (*CDC37*; lanes 1 and 2) and *cdc37-34* strain AFY14 (*cdc37*; lanes 3 and 4). Cultures incubated at 25°C were treated with (lanes 2 and 4) or without (lanes 1 and 3) 100 nM DHT for 1 hour. (B) As in A except that the cultures were incubated at 37°C for 1 hour prior to hormone administration and for 1 hour afterward. All results are the mean of three independent experiments.

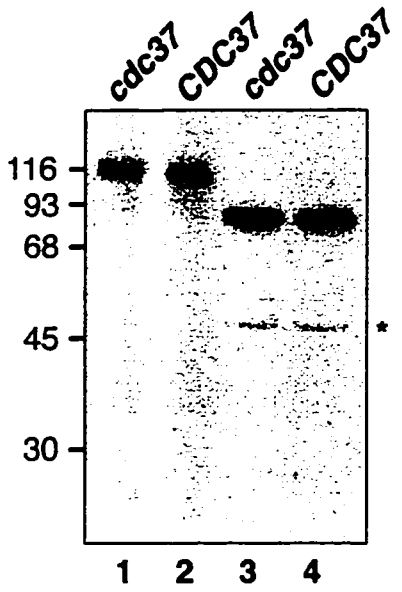


Figure 45. **Western Blot analysis of AR and AR^{1-600} in wild type and *cdc37-34* mutant yeast.** Western blot analysis of AR (lanes 1 and 2) and AR^{1-600} (lanes 3 and 4) in wild type strain AFY17 (*CDC37*; lanes 2 and 4) and *cdc37-34* strain AFY14 (*cdc37*; lanes 1 and 3). Analysis was performed using whole cell extracts (1 µg in lanes 1 and 2 and 5 µg in lanes 3 and 4) probed with anti-AR polyclonal antisera. Molecular weight size standards are shown in kDa. Star denotes breakdown product from AR^{1-600}.

Although a significant defect is seen in hormone dependent transactivation of the AR when comparing the wild type and the *cdc37-34* mutant strains, the mutant phenotype is only slightly suppressed (approximately two fold) when the wild type *CDC37* is expressed from a plasmid in the *cdc37-34* mutant background (Figure 46). Likewise, when a cross was made between the *cdc37-34* mutant strain and a wild type W3031a there was a similar defect in DHT induced lacZ gene expression as seen when the wild type plasmid is expressed in the mutant background at 25°C and 37°C (Figure 47-lanes 2 and 6), whereas, when the isogenic wild type was crossed with W3031a wild type lacZ expression was seen (Figure 47-lanes 4 and 8). This indicates that the *cdc37-34* allele is partially dominant negative over the wild type allele, since low copy number wild type Cdc37 expression was unable to restore LacZ activity of the *cdc37-34* mutant to wild type levels. It was only when the wild type gene was over-expressed from a GAL1 promoter on a multiple copy plasmid was wild type DHT induced lacZ gene expression restored (Figure 48). Expression in galactose was about 125 fold over that of the mutant protein grown in glucose as determined by Western blot (Figure 49). Although there seems to be some leakage of CDC37 gene expression in glucose, which can explain the increase lacZ gene expression in glucose at 25°C (Figure 49-lane 2). LacZ reporter gene assays were also performed on cdc37-34, cdc37-34/CDC37 and CDC37 yeast using R1881 (Figure 50). As seen in Figure 50, 100 nM R1881 was able to induce lacZ gene expression to levels similar to that of 100 nM DHT. Likewise, a transactivation

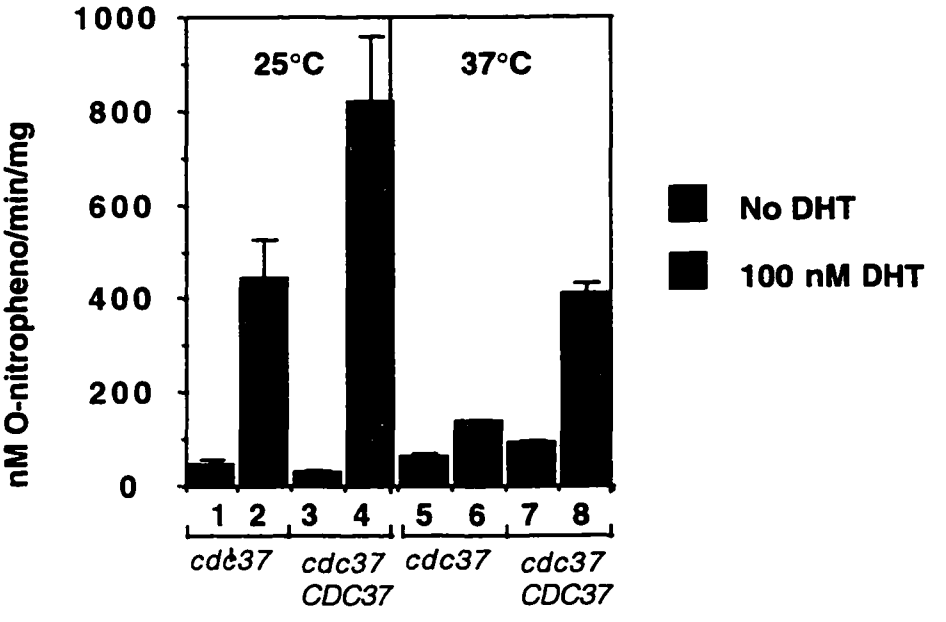


Figure 46. **The *cdc37-34* mutant allele acts dominant negative in AR signaling.** ß-Galactosidase activity in the *cdc37-34* mutant strain containing a low copy number vector strain AFY15 (pRS316; lanes 1,2,5 and 6) or a plasmid containing *CDC37* strain AFY16 (pRSS2; *cdc37/CDC37* in lanes 3,4,7 and 8). The cells were incubated with (lanes 2,4,6 and 80 or without (lanes 1,3,5, and 7) 100 nM DHT for 1 hour at 25°C or 37°C as indicated. All results are the mean of three independent experiments.

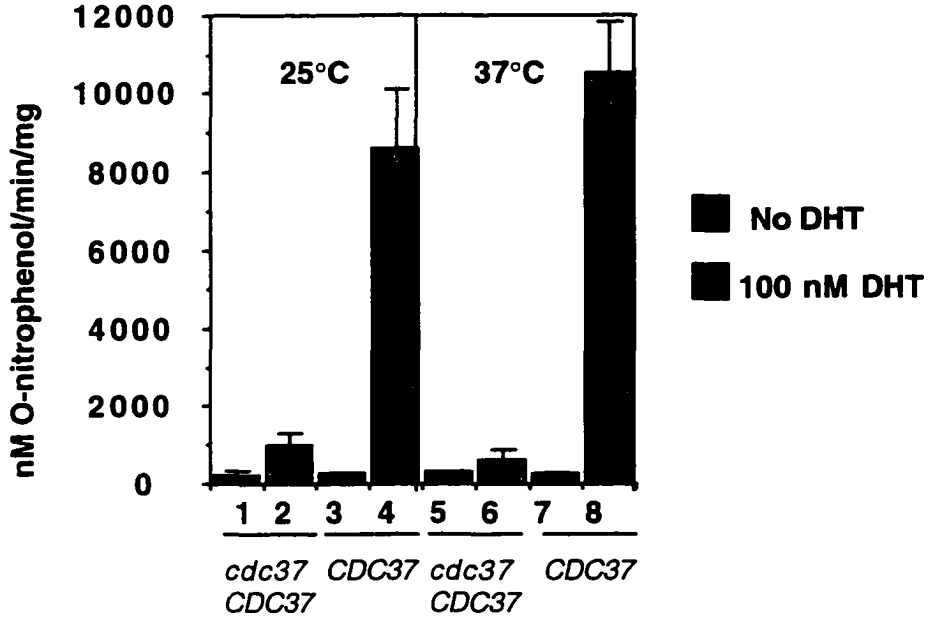


Figure 47. **Hormone dependent transactivation by AR is defective in a heterozygote *cdc37/CDC37* yeast strain.** ß-Galactosidase activity in *cdc37-34* crossed with *W3031a* (*cdc37/CDC37*; lanes 1, 2, 5, and 6) and *CDC37* crossed with *W3031a* (*CDC37/CDC37*; lanes 3, 4, 7, and 8). Cultures incubated at 25°C were treated with (lanes 2 and 4) or without (lanes 1 and 3) 100 nM DHT for 1 hour. Cultures incubated at 37°C were treated with (lanes 6 and 8) or without (lanes 5 and 7) 100 nM DHT. As for 25°C except that the cultures were incubated at 37°C for 1 hour prior to DHT administration and for 1 hour afterward. All results are the mean of three independent experiments.

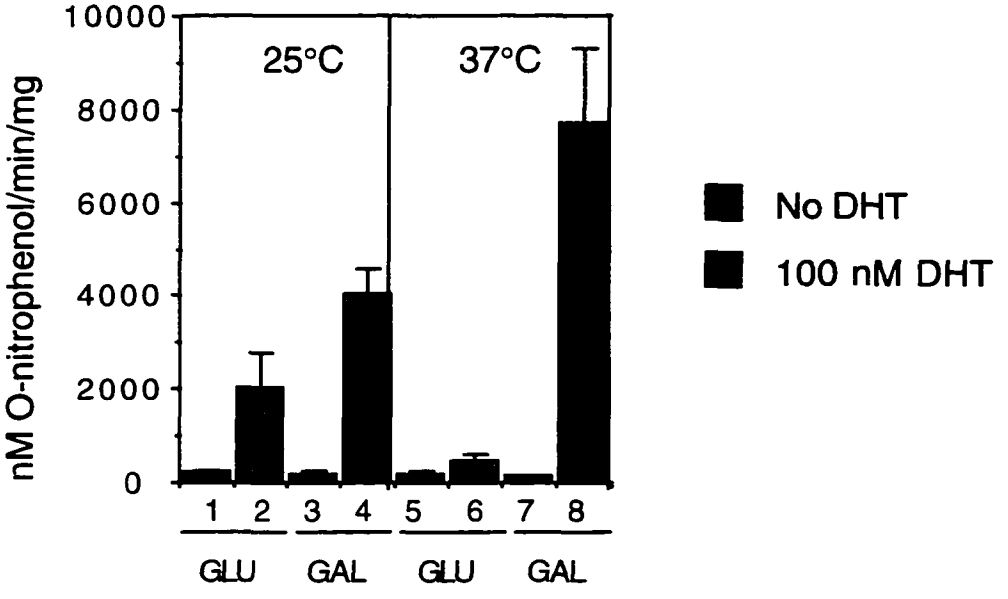


Figure 48. **Overexpression of *CDC37* in *cdc37-34* mutant yeast complements the AR signaling defect.** ß-Galactosidase activity in the *cdc37-34* mutant containing the multicopy 2µ plasmid containing *CDC37* under control of the inducible GAL1 promoter strain AFY18. Cells grown in glucose (GLU; lanes 1, 2, 5, and 6) or galactose (GAL; lanes 3, 4, 7, and 8) were incubated with or without 100 nM DHT at 25°C or 37°C as indicated. All results are the mean of three independent experiments.

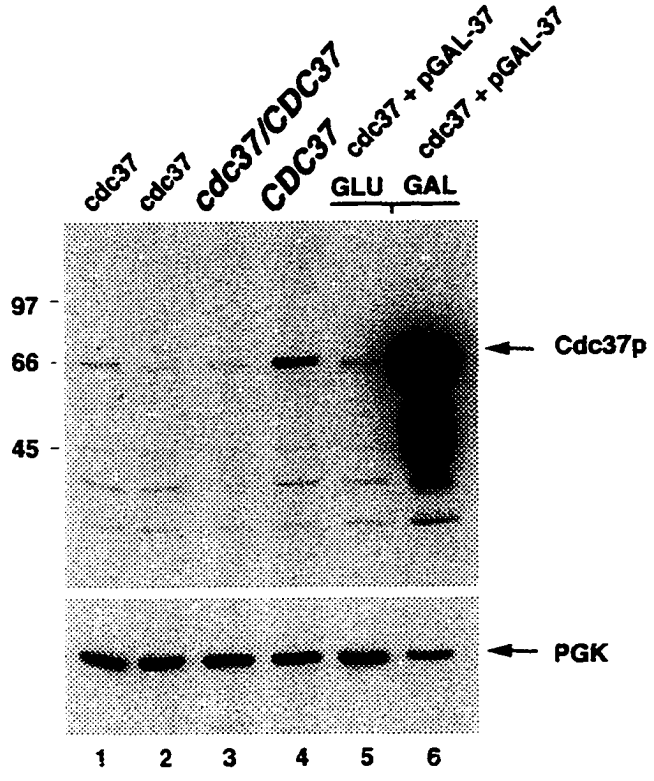


Figure 49. **Characterization of Protein Expression levels in *cdc37-34* mutant yeast.** Western Blot analysis of Cdc37 protein in whole-cell extracts from *cdc37-34* strain AFY14 (*cdc37*) grown at 25 °C (lane 1) or 37°C for 1 hour (lane 2), *cdc37-34* cells containing pRSS2 strain AFY16 (wild type *CDC37* on a low copy number plasmid) (*cdc37/ CDC37*; lane 3), wild type strain AFY17 (*CDC37*;lane 4), and *cdc37-34* cells containing pGAL-37 strain AFY18 grown in glucose (lane 5) or galactose (lane 6). Full length Cdc37p is arrowed. Bottom panel, reprobing the same filter with antisera against phosphoglycerate kinase (PGK; arrowed).

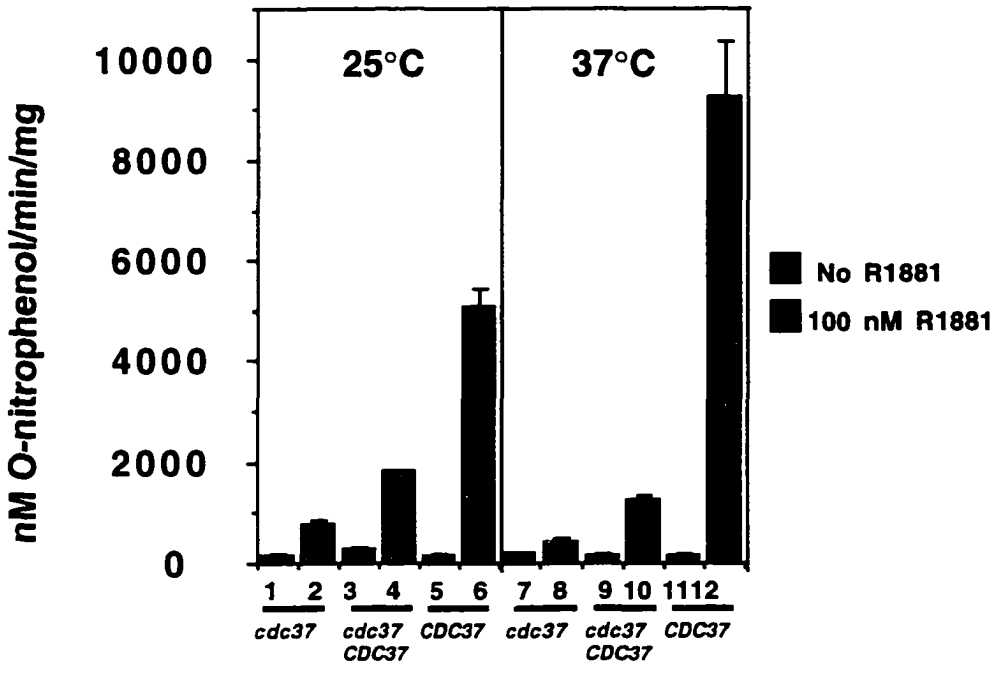


Figure 50. **R1881 dependent transactivation by AR is defective in a *cdc37* mutant strain.** ß-Galactosidase activity in *cdc37-34* strain AFY14 (*cdc37*; lanes 1, 2. 7 and 8), *cdc37-34* containing a *CDC37* plasmid strain AFY16 (*cdc37/CDC37*; lanes 3, 4, 9, and 10) and wild type strain AFY17 (*CDC37*; lanes 5, 6, 11, and 12) yeast. Cultures incubated at 25°C were treated with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) 100 nM R1881 for 1 hour. Cultures incubated at 37°C were treated with (lanes 8, 10, and 12) or without (lanes 7, 9, and 11) 100 nM R1881. As for 25°C except that the cultures were incubated at 37°C for 1 hour prior to R1881 administration and for 1 hour afterward. All results are the mean of three independent experiments.

defect was seen with R1881 in both the cdc37-34 and cdc37-34/CDC37 yeast strains that were heterologously expressing AR.

In order to further characterize the *cdc37-34* mutant phenotype, Cdc37 protein levels were determined using Western blot analysis of whole cell extracts (Figure 49). Extracts were prepared from wild type, mutant, mutant with wild type on a plasmid and mutant with wild type overexpressed from a GAL1 promoter. The level of Cdc37p was approximately 20 fold less in the *cdc37-34* mutant when compared to the wild type (Figure 49). In addition, the mutant protein seems to have somewhat of a dominant negative effect on the wild type protein, since there was a less than additive increase in Cdc37 protein levels when the wild type gene was expressed in low copy number in the *cdc37-34* mutant strain(Figure 49). Interestingly, these heterozygotes were able to grow at 37°C, albeit somewhat slower than the wild type. Overexpression of Cdc37p from a GAL1 promoter on a multiple copy plasmid also led to a decrease in cell growth at 37°C (Figure 42), when compared to the wild type, even though DHT induced transactivation was restored to wild type levels under the same conditions (Figure 48). This suggests that over-expression of Cdc37p might be deleterious to growth at higher temperatures.

[2] Cdc37p Function Does Not Affect AR Hormone Binding, Even Though Cdc37p Itself Acts Via the Hormone Binding Domain

Previous studies have shown that deletion of the HBD releases the AR from hormone dependence for transactivation (Simenthal et. al., 1991) Likewise,

factors that function via this domain are no longer able to regulate the AR in a truncation mutant which lacks the HBD (Nathan and Lindquist, 1995; Caplan et. al., 1995). For example, mutations in the YDJ1 gene which cause a defect in hormone dependent transactivation by the AR can be suppressed by deletion of the HBD (Caplan et al., 1995). In order to determine whether Cdc37p acts via the HBD a truncated AR (AR^{1-600}), which lacks the HBD, was constitutively expressed in both the wild type and *cdc37-34* mutant strains. Hormone independent lacZ reporter gene activity was measured for each of these strains at 25°C (the permissive temperature) (Figure 51). Levels of hormone-independent activity were similar when comparing the wild type and the *cdc37-34* mutant (Figure 51), indicating that Cdc37p acts via the HBD. This also demonstrated that mutation of Cdc37p does not affect lacZ reporter gene or AR^{1-600} folding or expression. Similar to the situation with full length AR, the truncated AR protein level was not altered in the *cdc37-34* mutant (Figure 45).

The chaperone machine associates with the HBD of the AR (Mariovet et al., 1992) and loss of Hsp90 function results in a decrease in high affinity hormone binding (Chapter 2 of this thesis; Fang et al., 1996). Since Cdc37p works in association with Hsp90, it is possible that Cdc37p is acting via the HBD in hormone binding. In order to determine whether Cdc37p is required for hormone binding to the AR, direct ligand binding assays were performed on both the wild type and *cdc37-34* mutant strains with ^{3}H R1881. These R1881 binding assays were performed at both the permissive (25°C) and

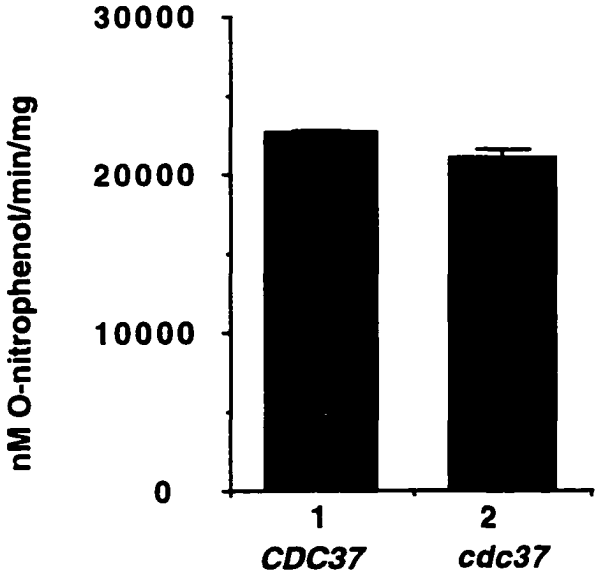


Figure 51. **Transactivation by AR^{1-600} in wild type and cdc37-34 mutant yeast strains.** Steady state ß-Galactosidase activity in wild type strain AFY17ABC (*CDC37*; lane 1) and cdc37-34 strain AFY14ABC (cdc37; lane 2) mutant strains constitutively expressing AR^{1-600}. Results are the mean of three independent experiments.

non-permissive (37°C) temperatures. Results from Figure 52 show that there is very little difference if any between the levels of hormone binding when comparing the wild type and *cdc37-34* mutant strains at either the permissive or non-permissive temperatures. This indicates that hormone binding to the AR is not defective in the *cdc37-34* mutant strain.

The fact that the *cdc37-34* mutant strain was not defective in AR hormone binding was confirmed using HF competition assays. As previously described, HF normally is a poor competitors of R1881 binding to the AR in wild type yeast, but in the absence of functional Hsp90 HF acts as a potent competitor (Chapter 2 of this thesis (Figure 19); Fang et al., 1996). In order to further characterize the phenotype of Cdc37p in hormone binding, ligand competition assays were performed on both wild type and *cdc37-34* mutant yeast using ^{3}H R1881 in the presence or in the absence of 25 uM HF and remaining R1881 binding was measured. Similar experiments were performed in parallel with the *G170D hsp82* mutant strain (Nathan and Lindquist, 1995) and were used as a positive control. As seen in Fig 53, very little competition was seen by HF in the *hsp82* mutant at the permissive temperature of 25°C, however, at the restrictive temperature 25 uM HF reduced R1881 to 20% of its original value. In contrast, in the *cdc37-34* strain HF acted as a poor competitor at both the permissive and non-permissive temperatures (Figure 53).

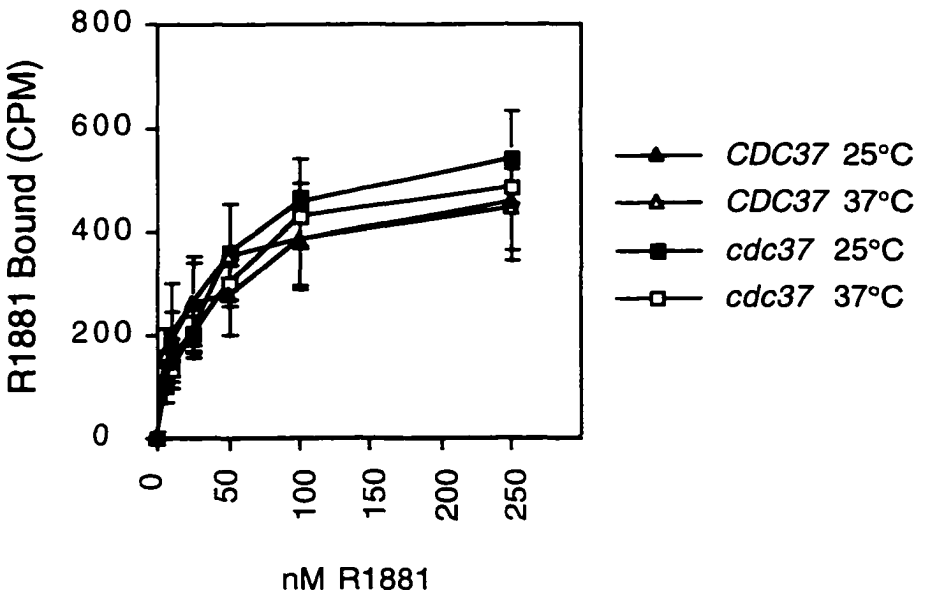


Figure 52. **Hormone binding to AR in wild type and *cdc37-34* mutant yeast strains.** Titration of 3H-R1881 in yeast cells expressing AR. Wild-type strain AFY17 (*CDC37*; squares) and *cdc37-34* mutant strain AFY14 (*cdc37*; triangles) were tested at 25°C (open symbols) and 37°C (closed symbols). Results are the mean of three independent experiments.

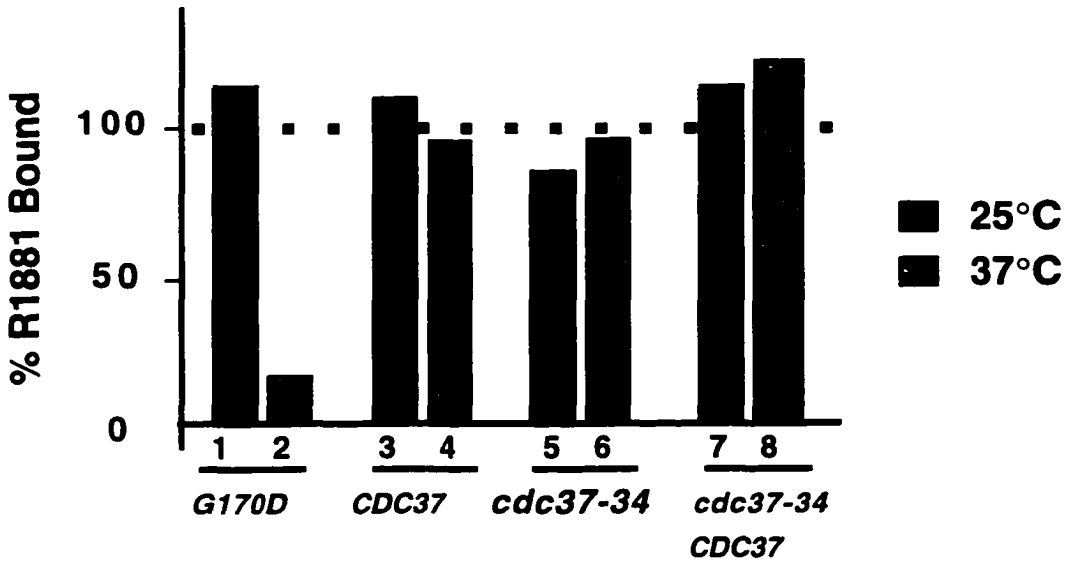


Figure 53. **HF competition assay on AR in *cdc37-34* mutant yeast**. *cdc37-34* strain AFY14 and *hsp82* mutant strain ACY99 were incubated at 25°C (lanes 1, 2, 5, and 6, respectively) or at 37°C (lanes 3, 4, 7, and 8, respectively) with (even lanes) or without (odd lanes) 25 μM HF in the presence of 100 nM 3H R1881. Results are expressed as a percentage of the 3H R1881 binding in the presence of HF. Results are the mean of three independent experiments.

[3] Cdc37p Differentially Regulates GR and ER Signaling

In order to determine whether Cdc37p plays a conserved role in hormone dependent activation of all steroid receptors, lacZ reporter gene assays were performed on wild type and *cdc37-34* mutants strains that were heterologously expressing either GR or ER. For the GR studies, full length rat GR was transformed into both wild type and mutant yeast containing the same reporter plasmid used for the AR studies, since GRE's (glucocorticoid response element) and ARE's (androgen response elements) are identical (Ham et al., 1988). As seen in Fig 54A, DOC induced lacZ gene expression in both the wild type (lane 2) and the *cdc37-34* (lane 4)to approximately the same level (approximately 30 fold over background) at the permissive temperature. These results were in contrast to the defect seen in AR signaling at the same temperature (Fig 44A). Even at the non-permissive temperature of 37°C, there was only a two fold decrease in deoxycorticosterone **(DOC)** induced lacZ reporter gene expression in the *cdc37-34* mutant (Figure 54-lane 4), compared to an 80 fold decrease seen for the mutant at the same temperature with the AR (Figure 44B). In addition, there was a slight decrease in lacZ levels at 37°C, compared to 25°C for the GR. This was in sharp contrast to the increase seen for the AR under the same conditions (Figure 44B). This indicates that GR signaling is only moderately defective in the *cdc37-34* mutant strain.

For similar studies with the ER, a plasmid encoding the full length human receptor and a separate plasmid containing an ERE lacZ reporter gene construct were co-transformed into both the wild type and *cdc37-34* mutant

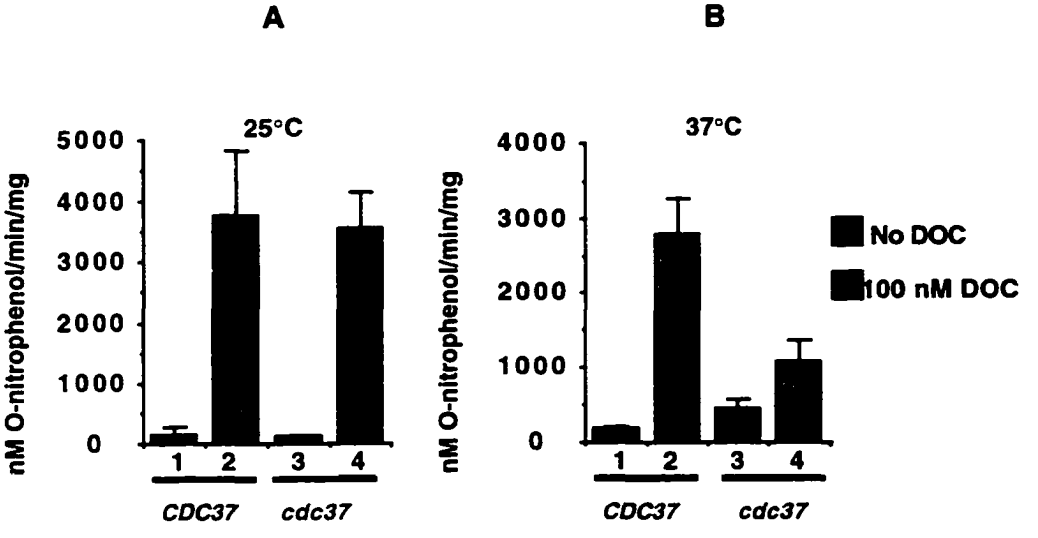


Figure 54. **Transactivation by GR in wild type and cdc37-34 mutant strains.** (A and B) ß-Galacatosidase activity in wild type AFY17GR (*CDC37;* lanes 1 and 2) and *cdc37-34* AFY14GR (*cdc37;* lanes 3 and 4) mutant strains containing GR. Cultures were incubated at 25°C (A) or 37°C (B) for 1 hour prior to addition of 100 nM DOC. Samples in lanes 1 and 3 contained no hormone. Results are the mean of three independent experiments.

strain. LacZ reporter gene assays were performed on both strains in the presence or absence of 100 nM E2. As seen in Fig 55, E2 induced lacZ gene expression was similar in both wild type and *cdc37-34* mutant strains at the permissive temperature (Figure 55A) and there was less than a 50% decrease in the mutant at the non-permissive temperature (Figure 55B), compared to the wild type at the same temperature. Similar to GR, ER signaling is only modestly defective in the absence of functional Cdc37p.

Discussion

In this chapter, it has been demonstrated that the yeast CDC37 gene has differential function in the regulation of steroid hormone receptor activation. In the *cdc37-34* mutant strain, hormone dependent activation of the AR is dramatically reduced at both the permissive (Figure 44A) and non-permissive temperature (Figure 44B). This defect is dependent upon the presence of the HBD (Figure 51), suggesting that Cdc37p acts via this domain. However, there is no hormone binding defect in the *cdc37-34* strain, compared to the wild type strain (Figure 52 and 53). In contrast to the AR, GR and ER hormone dependent signaling is only moderately affected at the non-permissive and identical to the wild type at the permissive temperature (Figure 54 and 55). The *cdc37-34* allele was also partially dominant negative with respect to AR activation (Figure 46) and wild type Cdc37p levels (Figure 49). So AR signaling may require higher levels of Cdc37p for full function, whereas GR and ER can function reasonably at lower Cdc37p levels. This suggests that

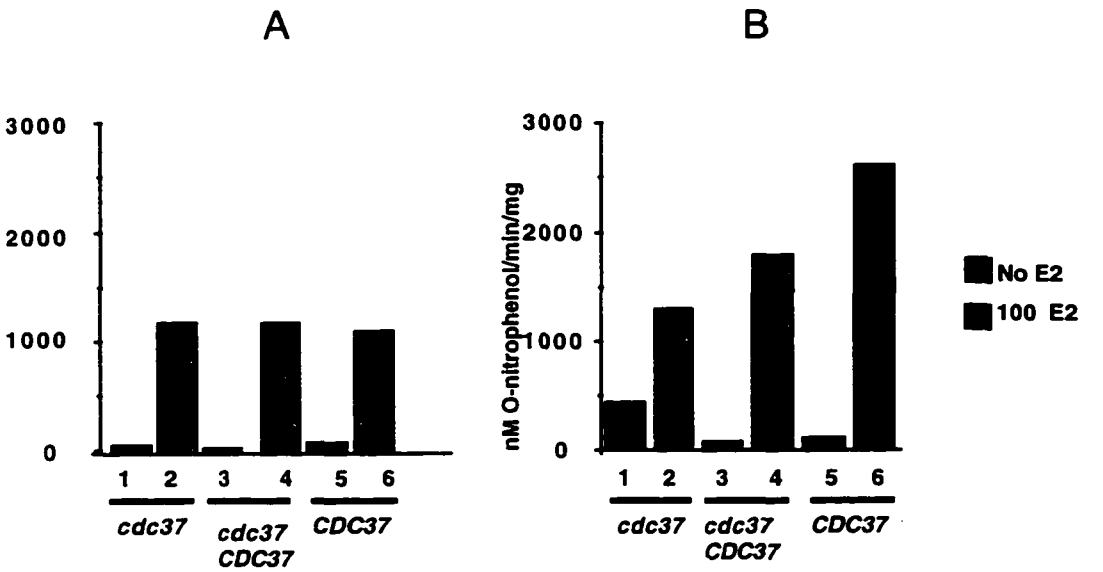


Figure 55. **Transactivation by ER in wild type and *cdc37-34* mutant strains.** (A and B) ß-Galacatosidase activity in *cdc37-34* strain AFY14ER(*cdc37*; lanes 1 and 2), *cdc37-34* containing pRSS2 strain AFY16ER (low copy number plasmid that constitutively expresses wild type Cdc37p (*cdc37/CDC37*; lanes 3 and 4) and wild type strain AFY17ER (*CDC37*; lanes 5 and 6) mutant strains containing ER. Cultures were incubated at 25°C (A) and 37°C (B) for 1 hour prior to treatment. Cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 100 nM E2.

Cdc37p is involved in the hormone dependent activation of many steroid hormone receptor, but to differing extents.

Similar receptor specific differences in hormone dependent activation have also been seen with Hsp90. In previous studies, GR activation was severely compromised in *hsp82*^{G170D} mutant yeast (Nathan and Lindquist, 1995), whereas in the same mutant strain hormone dependent activation of the AR was only reduced 2-3 fold at saturating concentration of hormone (Fang et al., 1996). Similarly, activation differences have been seen among other *hsp82* mutants which were defective for receptor signaling. One of the most extreme examples is that of the E431K mutant, in this mutant hormone dependent GR signaling was severely reduced, whereas, ER, MR and PR signaling was not affected (Bohen and Yamomoto, 1993). In contrast, hormone dependent activation of all four receptors was severely compromised in the G313N mutant (Bohen and Yamamoto, 1993). These differences as well as the one seen with *cdc37-34* may reflect upon distinct properties related to the activation of each receptor.

The results presented here are the first conclusive demonstration for the function of Cdc37p in steroid hormone receptor function. Several previous reports have demonstrated that Cdc37p functions only in protein kinase activation pathways (Gerber et al., 1995; Valay et al., 1995; Dey et al., 1996; Stepanova et al., 1996). Since the only previously demonstrated role for Cdc37p has been in protein kinase activation, this raises the question as to whether Cdc37p might act indirectly on AR via a protein kinase that is required

for hormone dependent function. However, it has been previously demonstrated using transfection studies in mammalian cells that deletion of all three major phosphorylation sites in the AR results in only a 30% decrease in AR transactivation (Jenster et al., 1994; Zhou et al., 1995). These previous results would infer that phosphorylation does not play a major role in hormone dependent activation of the AR, since loss of Cdc37p function results in a greater than 90% decrease in receptor function . This leads us to postulate that Cdc37p might interact directly with the AR or Hsp90 is the AR aporeceptor complex itself.

It is unclear exactly how Cdc37p might fit into the stepwise pathway to the activation of AR, since loss of its function has no effect on high affinity hormone binding and it does not seem to associate stably with steroid receptors. This suggests that Cdc37p might play a role downstream of hormone binding, but prior to DNA binding and transactivation, since Cdc37p is not required for hormone independent transactivation. This leads one to postulate that Cdc37p might be involved in the conversion of the hormone bound receptor into its active form. This conversion may involve conformational changes in the HBD post-hormone binding that are needed for downstream receptor function. Little if any is known about these types of hormone initiated allosteric changes, but it remains possible that Cdc37p might act as a molecular chaperone and facilitate these alterations upon ligand binding. Interestingly, Kimura et al. (1997) recently demonstrated that Cdc37p can act as a molecular chaperone *in vitro* by stabilizing the partially folded form of a polypeptide (Kimura et. al.,

1997). It is therefore possible that Cdc37p exerts similar effects on the HBD in hormone dependent activation of steroid receptors.

Experimental Methods

Yeast Strains and Growth Conditions

Yeast cells were cultured in selective media (0.67% yeast nitrogen base, 2% glucose or galactose plus the appropriate amino acids) using standard procedures. The temperature-sensitive strain 8A7 (MATα *cdc37-34*, leu2, lys2, trp1, ura3) was used as the parental strain for this study. Plasmid transformations were performed by the LiAc procedure as described previously (Geitz et al., 1995). Plasmids used in this study were: pG1-hAR (human AR); Caplan et al., 1995), pABC (AR^{1-600}, TRP1; Caplan et al., 1995), pGAL-37 (GAL1 promoter, CDC37, LEU2), pPGKareLacZ (lacZ reporter gene under control of androgen response elements, URA3; Purvis et al., 1991), pPGKgal-hAR (galactose-inducible AR gene, URA3; Purvis et al., 1991), pGN795 (rat GR, TRP1; Schena and Yamamoto, 1988), pRSS2 (CDC37, CEN/ARS, URA3; Dey et al., 1996) and pRS315-CDC37 (CDC37, CEN/ARS, LEU2), p2HGPDER/CYC (human ER, 2μ, HIS3; Kimura et al., 1995), pUCΔSS-ERE (lacZ reporter gene under control of estrogen response element, URA3; Picard et. al., 1990) and pJR3 (human ER, 2μ, TRP1; Chapter 2 of this thesis).

The isogenic wild type strain was constructed by transforming a 6-kb DNA fragment containing the wild type CDC37 (Sal1/Hind111 digestion of pRSS2

(Dey et al., 1996) into AFY14 (8A7 with pG1-hAR and pPGKareLacZC) and colonies were selected for growth at 37°C. The resulting strain was called AFY17. Gene replacement of the mutant allele was confirmed using direct genomic DNA sequencing (sequence analysis performed by Dr. Robert Donnelly, UNJ). Strains containing pABC (Truncated AR), pPGKgalhAR (AR under galactose control) , pGN795 (GR) and p2HGPDER/CYC (Picard et. al., 1990) were prepared after deselection of pG1hAR (AR) from AFY14 and AFY17 on non-selective media and subsequent transformation by these plasmids. The resulting strains that contained pABC, pPGKgalhAR or pGN795 all contained the pPGKarelacZC reporter plasmid, whereas, the strains containing p2HGPDER/CYC were subsequently transformed with pUCΔSS-ERE. The *hsp82*^{G170D} mutant strain used for the sake of comparison was described previously (Nathan and Lindquist, 1995; Fang et al., 1996; Chapter 2 of this thesis).

ß-Galactosidase Activity Assays

Yeast cells were grown to early log phase and preincubated at either 25°C or 37°C for 1 hour before addition of DHT for the AR, DOC for the GR and E2 for the ER. The cells were subsequently incubated for another hour at the same temperature prior to the preparation of extracts as described previously (Caplan et al., 1995). Following extract preparation samples were assayed for ß-Galactosidase activity as described previously by Caplan et al. (1995).

Ligand Binding Assays

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 minutes. Following this preincubation, cells were incubated with ^{3}H R1881 for an additional 1.5 hours at the same temperature. The cells were then washed 3 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 R1881 from the samples incubated in the absence of cold R1881.

Ligand competition assays were also performed with yeast cells which were grown to early log phase (OD^{600}= 0.2) as described above. Following a 30 minute preincubation at either 25°C or 37°C, cells were incubated with 100 nM ^{3}H R1881 in the presence or absence of varying concentrations of HF for an additional 1.5 hours at the same temperature. The cells were then washed 3 times with 1 ml of water each and counted in 5 ml of scintillation fluid.

Western Blot Analysis

The levels of AR and Cdc37p were assayed by Western blot analysis using either anti-AR or anti-Cdc37p specific antibodies. Yeast lysates were prepared as previously described (Caplan et. al., 1995) Lysates (5 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 subsequently incubated with polyclonal antibodies specific for the AR or monoclonal antibodies specific for yeast Cdc37p (antibodies were diluted in antibody dilution buffer, 1x PBS, 3% bovine serum albumin, 0.05% Tween 20 and 0.1% thimerosal (1:1000 for anti-Cdc37p and 1:1000 for anti-AR) for 1 hour. Filters were washed three times for 10 minutes each in TTBS. Filters were then incubated with secondary antibody (HRP conjugated goat anti-rabbit IgG, diluted 1:10,000 in antibody dilution buffer for anti-ER; and HRP conjugated goat anti-mouse IgG, diluted 1:10,000 in antibody dilution buffer for anti-Cdc37) for 1 hour and subsequently washed three times for 10 minutes each in TTBS. Filters were treated with the chemiluminescence reagent (Pierce) and exposed to X-ray film. An identical filter was probed with anti-PGK (1:300,000 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-Cdc37p and anti-AR.

CHAPTER V

OVERALL DISCUSSION AND FUTURE DIRECTION

It has been long known that Hsp90 is present in the aporeceptor complex of all members of the steroid hormone receptor superfamily including the AR and ER, although little was known of its function in vivo (Mariovet et al, 1992; Redeuilh et al, 1987; Nemoto et al, 1992; Schuh et al, 1985; Radanyi et al, 1989; Sanchez et al, 1985 and Rafestin-Oblin et al, 1989). There has been a recent resurgence in the study of the function of Hsp90. This renewed interest in the function of Hsp90 was sparked from the discovery that Hsp90 itself is a target for a group of anti-tumor agents called the benzaquinoid ansamycins (Whitesell et. al., 1994). This group of anti-tumor compounds includes MacBecin 1, MacBecin 2 and geldanamycin. Hsp90 has been recently crystallized in the presence of geldanamycin (Stebbins et. al., 1997). The crystal structure revealed that geldanamycin itself is able to interact and perhaps block binding of ATP to the ATP binding domain of Hsp90. Likewise, as expected geldanamycin can specifically inhibit by direct binding many of the signal transduction pathways that Hsp90 is known to regulate including that of several protein kinases (Uehara et. al., 1988) and steroid hormone receptors (Smith et. al., 1995; Segnitz and Gehring, 1997).

Previous studies suggested that Hsp90 is required for high affinity hormone binding to the GR (Picard et. al., 1990; Bresnick et. al., 1989; Bohen, 1995), PR (Smith, 1993) and MR (Schulman et. al., 1992). These studies have been performed both *in vitro* and *in vivo*. *In vitro* studies suggest that GR and MR have strict requirements for Hsp90, since in its absence neither receptor is able

to bind hormone. Likewise, results from *in vivo* studies using the yeast model system confirm the requirement of Hsp90 function in high affinity hormone binding to the GR (Bohen, 1995) and MR (Schulman et. al., 1992). In contrast, PR has somewhat of a modified requirement for Hsp90 (Smith, 1993) PR is able to bind hormone at 4°C in the absence of Hsp90, but when these same experiment are performed at 37°C PR is unable to bind hormone in the absence of Hsp90. Until now it was thought that AR and ER had no requirement for Hsp90 in hormone binding. *In vitro* studies had previously suggested that AR and ER could bind hormone in the absence of Hsp90 association (Nemoto et. al, 1992; Binart et. al., 1995). The caveat being these experiments were performed at non-physiological temperature *in vitro*, conditions in which molecular chaperone may not normally be required. Since molecular chaperones themselves are needed to block off-pathway inter and intra-molecular interactions under conditions of high protein concentration and high temperature. However, typical in vitro experiment is performed at low temperature under low protein concentrations. Therefore it is advantageous to perform such experiment with molecular chaperones in vivo under physiological conditions.

In the study presented here, the yeast system was utilized to determine whether Hsp90 itself is needed for hormone binding to the AR and ER *in vivo*. The results presented here demonstrate that Hsp90 is required for both full activation and high affinity hormone binding to the AR and ER. In the absence of functional Hsp90, both the AR and the ER are unable to bind hormone to the

same extent as under wild type conditions (Figure17-19, 9) . Segnitz and Gehring also demonstrated that there was a similar decrease in ER hormone binding when animal cells were treated with the anti-Hsp90 agent, geldanamycin (Segnitz and Gehring, 1997).

Recent studies on the crystal structure of ER demonstrate that both E2 (hormone) and raloxifene (so-called partial antagonist) interact with similar residues in the hydrophobic hormone binding domain (Brzozowski et. al., 1997). Likewise in the studies described here, there was also an alteration in the manner in which so called hormone antagonist were able to interact with both the AR and ER, which would be expected since both ligands are interacting with the HBD. In the case of the AR, the normally weak competitive inhibitor HF acted as a more potent inhibitor of hormone binding in the absence of Hsp90 function (Figure 19) Whether this was due to decrease in hormone binding affinity or an increase in the affinity of HF is not known. In contrast, 4-OHT was actually able to increase the binding of DES (hormone) to the ER in the absence of functional Hsp90 (Figure 5, 8)), although this increase in hormone binding did not lead to concurrent stimulation of transactivation (Figure 16) Taken together these data suggest that Hsp90 is required for proper function and hormone binding of both the AR and ER *in vivo.* This would place the AR and the ER in a similar category as PR for their requirement for Hsp90, since all three receptors are able to function in the absence of Hsp90 in non-physiological conditions. The importance of Hsp90

has also been suggested by the fact that naturally glucocorticoid resistant leukemia cells are significantly decreased in Hsp90p (Kojika et. al., 1996).

As previously described, Hsp90 does not exist on its own in the aporeceptor complex. It is joined with several co-chaperones of which several are binding proteins of Hsp90. Therefore it is crucial to separate the role of Hsp90 itself from its co-chaperones in steroid receptor hormone binding and action. Does Hsp90 itself function to allow for hormone dependent receptor function or is this the function of one of the several co-chaperones found in the unliganded receptor complex? In order to determine this the role of each member of this complex must be analyzed individually.

Hsp70 is one of the best characterized components of the Hsp90 chaperone machine. Hsp70 is able to assist in the folding of newly synthesized polypeptide chains coming off the ribosome (Gething and Sambrook, 1993). Likewise, Hsp70 itself has been implicated to play a role in signaling by the GR *in vitro* (Hutchison et. al., 1994; Smith et. al., 1994). Upon depletion of Hsp70, Hsp90 is not present in the aporeceptor complex and GR is unable to bind hormone, suggesting that Hsp70 is required for hormone binding. This is probably due to the fact that Hsp90 is not recruited into the unliganded receptor complex in the absence of Hsp70. Hsp70 depends on the presence of ATP for its function in protein folding (Liberek et. al., 1991). Hsp70 is able to bind ATP and in its ATP bound form is unable to stably interact with its polypeptide substrates. Upon ATP hydrolysis, Hsp70-ATP is converted to Hsp70-ADP and in this form is able to stably interact with its polypeptide

substrates. Thus Hsp70 must be able to efficiently hydrolyze ATP to ADP and subsequently exchange nucleotides to continue the cycle. In order to accomplish this task, Hsp70 is aided by other components of the chaperone machine, including in these is Ydj1p.

Ydj1 is able to stimulate the low level ATPase activity of Hsp70 (Tsai and Douglas, 1996) and has general chaperone function of its own via its zinc finger and carboxy-terminal domains (Lu and Cyr, 1998). Ydj1p is considered a Type 1 J protein, since it contains the J G/F and zinc finger domains. It had been previously demonstrated that yeast containing a mutant form or deletion of the Ydj1p are defective for AR and ER signaling (Caplan et. al., 1995; Kimura et. al., 1995). In the case of the AR there was a dramatic decrease in hormone induced signaling which was dependent upon the presence of the HBD (Caplan et. al., 1995). Therefore, it was postulated that this defect may be in the ability of AR to bind hormone in the absence of Ydj1p.

In vivo yeast studies presented here demonstrate that the decrease in AR activation seen in the *ydj1* mutant yeast is indeed caused in part by a defect in hormone binding (Figure 24). AR is unable to bind hormone at wild type levels in yeast deleted for or containing a mutation in Ydj1p. Loss of J domain function resulted in a decrease in AR hormone binding and transactivation (Figure 27 and 34). This suggest not only that the J domains essential for hormone dependent activation, but also that Ydj1 is more that likely acting via Hsp70 in AR signaling. Likewise, loss of the zinc finger domain and/or farnesylation also results in a decrease in AR hormone binding and

transactivation (Figure 27 and 34), suggesting that either or both of these regions act in conjunction with the J domain to allow for wild type AR signaling. The only domain that is clearly dispensable for hormone binding in the carboxy-terminal domain of Ydj1, since the ydj1-G315D mutant (which is mutated in the carboxy-terminal domain) is not defective for hormone binding to the AR (Figure 30).

Under normal conditions *in vivo* , the AR does not interact with the yeast chaperone Ydj1p, more appropriately it would interact with the human homologue of Ydj1p. Hdj2p is a human homologue of Ydj1 and is also a Type 1 J protein (For review see Cheetham and Caplan, 1998). The question posed in the study presented here is "Can a human homologue of Ydj1 complement the AR signaling defect of the Ydj1 null strain?". Of the three human J proteins tested, it would be expected that only Hdj2p would be able to fully complement the AR signaling defects. As expected neither Type 2 J protein (contains J G/F domains), Hsj1p nor Hdj1p, was able to complement either the hormone binding or transactivation defects. Only Hdj2p was able to fully complement the hormone binding, ligand competition and transactivation defect. Yeast deleted for Ydj1p regained essentially wild type hormone binding (Figure 36), ligand competition (Figure 38) and hormone dependent transactivation (Figure 39) when transformed with a Hdj2p expression plasmid. This demonstrates that a human molecular chaperone can work in association with yeast chaperones to allow for proper regulation of a human steroid receptor. Likewise the human ER was defective in hormone dependent signaling in the

same *ydj1* mutant yeast. The importance of Ydj1p is steroid receptor signaling has also been recently confirmed using an in vitro reconstitution system (Dittmar et. al., 1998) (Figure 40-41). It was shown that depletion of Hsp40 caused a decrease in hormone binding to the GR and this defect was able to be complemented by the addition of Ydj1p (Dittmar et., al., 1998). Taken together these results suggest that Ydj1p, or more correctly the human homologue Hdj2p is required for steroid hormone receptor signaling probably via Hsp70. These results also suggests that Type 1 J proteins may act via Hsp70 in recruiting Hsp90 into the aporeceptor complex, placing its role upstream of Hsp90 in the attainment of the high affinity hormone binding conformation.

Other components of the chaperone machine that are seen in association with Hsp90 in the unliganded receptor complex are p60/Sti1, p23/Sba1, p48/HIP and one of the many cyclo/immunophilins. Of these components, p60 is thought to be the nucleotide exchange factor of Hsp70 and has been demonstrated to play a minor role in GR signaling in vivo using yeast as a model organism (Chang et. al., 1997). Upon deletion of Sti1, yeast demonstrate a modest decrease in hormone dependent transactivation. Likewise, the cyclophilin Cpr7 has also been demonstrated to play a role in GR signaling. Again a modest decrease in hormone dependent transactivation is seen in a *Δcpr7* yeast strain (Duina et. al., 1996). However, in similar studies Picard et al., was unable to see a GR, ER or PR signaling defect upon deletion of the cyclophilin Cyp40/Cpr6 (Warth et. al., 1997). This suggests that Cpr7,

but not Cpr6 plays a role in receptor signaling. Of the co-chaperones previously described, only p23/Sba1 has not been shown to play an important role *in vivo* in steroid receptor signaling, even though, p23 is essential for high affinity hormone binding to the GR and PR *in vitro* in rabbit reticulocyte lysates (Hutchison et. al., 1995; Hutchison et. al., 1994; Johnson et. al., 1994; Johnson and Toft, 1995; Smith et. al., 1990a). GR and AR hormone dependent signaling is essentially wild type in the absence of the SBA1 gene product, suggesting that p23 plays no essential role in receptor signaling *in vivo* (Fang et. al., 1998; Bohen, 1998).

Cdc37p is also able to specifically bind Hsp90 (Whitelaw et., 1991; Brugge et. al., 1986) and was postulated to be the protein kinase targeting component of the Hsp90 chaperone machine. Cdc37p is essential for CDK4 and CDK6 (Dai et. al., 1996; Stepanova et. al., 1996), Cdc28 (Gerber et. al., 1995), *Drosophila* Sevenless (Cutworth and Rubin, 1994), v-src (Dey et. al., 1996) signaling pathways, to mention a few. Until recently it was thought that p50/Cdc37p was only essential for protein kinase signaling via the Hsp90 chaperone machine, since it is not seen in the aporeceptor complex of either ER or GR (Nair et. al., 1997). However, studies presented here are to the contrary and are the first demonstration of Cdc37p's role in steroid hormone receptor signaling. In the absence of functional Cdc37p, AR had a profound decrease in hormone dependent transactivation (Figure 44), whereas, GR (Figure 54) and ER (Figure 55) signaling was only moderately effect upon loss of function. This suggest that Cdc37p differentially affects steroid hormone

receptors. Previous studies have shown similar differential affects with the E431K *hsp82* mutant, E431K dramatically decreased GR hormone binding and subsequent transactivation, whereas, ER, PR and MR signaling was slightly affected (Bohen and Yamamoto, 1993). Interestingly, the AR signaling defect seen in the Cdc37p mutant was dependent upon the presence of the HBD (Figure 51), even though, there was no alteration in hormone binding (Figure 52) or ligand competition (Figure 53). Since, previous studies have shown that AR is a phosphoprotein and since Cdc37p is thought to be the protein kinase targeting component of the chaperone machine one might postulate that Cdc37p is acting on a protein kinase which in turn is phosphorylating the AR. However, previous studies have demonstrated that the three main phosphorylation sites in AR can be mutated and there is only a 30% decrease in hormone dependent transactivation, suggesting that Cdc37p must be acting either via Hsp90 or the AR itself (Jenster et. al., 1994; Zhou et. al., 1995). This suggests that Cdc37p may be acting via the HBD after hormone binding possibly facilitating conformational changes that occur in the HBD upon ligand binding, placing Cdc37p after Hsp90 in the pathway to steroid receptor activation.

To date it has been demonstrated using either *in vitro* or *in vivo* model systems that Hsp90 (Picard et. al., 1990; Bohen and Yamamoto, 1993; Bohen, 1995; Bresnick et. al., 1989), Hsp70 (Hutchison et. al., 1994), Ydj1 (Caplan et. al., 1995; Kimura et. al., 1995), p50/Cdc37 (Fliss et. al., 1007), p60/Sti1 (Chang et. al., 1997), cyclophilins (Duina et. al., 1996) and p23/Sba1 (Hutchison et. al.,

1995) are essential for steroid receptor signaling. In order to fully understand the physiological role each of these individual components of the Hsp90 chaperone machine plays in receptor signaling, mammalian *in vivo* model systems must be developed to assess their role in their native environment. Recently two naturally occurring model system have been identified in which steroid receptor signaling is aberrant. In both of these systems, the defect is not in the steroid receptor itself, but in components that help regulate receptor function. One of these systems is a glucocorticoid resistant leukemia cell line that is unaffected or minimally affected by the presence of glucocorticoids (Kojika et. al., 1996).. Interestingly, both Hsp90 and Hsp70 are abnormal in either levels or composition in these leukemic cell lines. These cells could be utilized to determine whether Hsp90 or Hsp70 are required for receptor signaling in its native environment. In such studies wild type Hsp90 or Hsp70 could be overexpressed in order to determine whether the GR signaling defect could be complemented. If overexpression of either Hsp90 or Hsp70 are able to restore normal GR signaling this would be direct evidence of their role in steroid receptor signaling.

The second mammalian *in vivo* model is that of the glucocorticoid resistant squirrel monkey GR (Reynolds et. al., 1997). Again in this system the GR is unable to be activated except at high concentrations of glucocorticoid. This defect is not an aberration in the receptor itself, but an alteration in a regulatory factor. This system can be exploited and other components of the chaperone

machine can be transfected in these cells in order to determine their role in receptor signaling.

An alternative approach would be to utilize the functional conservation in the chaperone machinery to develop dominant negative mutants in the yeast system. And because of the conservation of the chaperone machine, these yeast dominant negative mutants could be transformed into human cells that normally express either the AR (LNCaP-human prostate cancer cell line) or the ER (MCF-7-human breast cancer cell line) in order to specifically block function of individual components of the chaperone machine. The constructs could be expressed under the control of inducible promoter in order to conditionally regulate the production of the dominant negative chaperone protein. Subsequently, hormone binding and transactivation studies could be performed of these cell lines that are expressing dominant negative mutants of each of the chaperone machine components. Dominant negative mutants for Hsp90 (Louvion et. al., 1996) and Cdc37 (Fliss et. al., 1997) have already been reported in the literature and constructs for the other co-chaperones could easily be made.

The use of the these mammalian model systems will allow for the further dissection and confirmation of results previously acquired using *in vitro* and yeast model systems and should further the understanding of the role of each component of the Hsp90 chaperone machine in steroid hormone receptor function. The understanding of the differential regulation of these receptors could also eventually lead to the development of targeted therapeutics.

CHAPTER VI

APPENDICES

Appendix I

List of plasmids

Plasmid name	Genotype	Reference
pts38RV	hsp82 ^{A97I}/CEN/ARS/HIS3	Kimura et. al., 1994
pcs2-3RV	hsp82^{T101I}/CEN/ARS/HIS3	Kimura et. al., 1994
pts33BE	hsp82^{S485Y}/CEN/ARS/HIS3	Kimura et. al., 1994
pTCA/hsp82 E431K	hsp82^{E431K}/CEN/ARS/TRP1	Bohen & Yamamoto, 1993
pTCA/hsp82 G313N	hsp82^{G313N}/CEN/ARS/TRP1	Bohen & Yamamoto, 1993
pTCA/hsp82 T525I	hsp82^{T525I}/CEN/ARS/TRP1	Bohen & Yamamoto, 1993
pG1-hAR	hAR/2μ/TRP1	Caplan et. al., 1995
pARU	hAR/2μ/URA3	Caplan et. al., 1995
pARH	hAR/2μ/HIS3	Caplan et. al., 1995
p2HGPDER/CYC	hER/2μ/HIS3	Picard et. al., 1990
pJR3	hER/2μ/TRP1	Cloned by Jie Rao
pJR1	HSP82/CEN/ARS/URA3	Fang et al.,1998
pUCΔSS-ERE	LacZ/2μ/URA3	Picard et. al., 1990
pPGKareLacZC	LacZ/CEN/ARS/URA3	Purvis et al., 1991
pYX233hsp40	HDJ1/GAL1/HIS3	
pGALHDJ2	HDJ2/GAL1/CEN/ARS/LEU2	Cloned by Jie Rao
pGALHSJ1	HSJ1/GAL1/CEN/ARS/LEU2	This thesis
pCB338	SIS1/2μ/URA3	Caplan and Douglas, 1991
pAV151	ydj1-151/CEN/ARS/LEU2	Caplan et al., 1992
pGALYDJ1	YDJ1/GAL1/CEN/ARS/URA3	Caplan et. al., 1992
pAV7	YDJ1,CEN/ARS/LEU2	Caplan et al., 1992)

pJR4	LacZ/CEN/ARS/LEU2	Cloned by Jie Rao
ydj1-H34QpRS315	ydj1-H34Q/CEN/ARS/LEU2	Cloned by Jie Rao
pABC	AR^{1-600}/2μ/TRP1	Caplan et al., 1995
pGAL-37	CDC37/GAL1/CEN/ARS/LEU2	Fliss et. al., 1997
pPGKgal-hAR	AR/GAL1/2μ/URA3	Purvis et al., 1991
pGN795	rGR/2μ/TRP1	Schena & Yamamoto,1988
pRSS2	CDC37/CEN/ARS/URA3	Dey et al., 1996
pRS315CDC37	CDC37/CEN/ARS/LEU2	This thesis

Appendix II

List of Strains

Strain Name	Genotype
W3031a	**a** *ade2 leu2 his3 trp1 ura3 can1*
P82a	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTGPDHsp82
G170Da	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTGpd/T1-101
ACY98	P82a with pARH
ACY99	G170Da with pARH
ACY 2W	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTGPDHsp82 and pJR1
ACY 1WU	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pJR1
AFY43	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTGPDHsp82, pUCΔSS-ERE and p2HGPDER/CYC
AFY44	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTGpd/T1-101, pUCΔSS-ERE and p2HGPDER/CYC
AFYA97I	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pts38RV
AFYT101I	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pcs2-3RV
AFYE431K	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTCA/hsp82 E431K
AFYS485Y	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pts33BE
AFYT525I	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTCA/hsp82 T525I
AFYG313N	**a** *ade2 leu2 his3 trp1 ura3 can1* (*hsc82*:: *LEU2*) (*hsp82*:: *LEU2*) pTCA/hsp82 G313N
AFYA97IER	AFYA97I with pJR3 and pUCΔSS-ERE
AFYT101IER	AFYT101I with pJR3 and pUCΔSS-ERE

AFYE431KER	AFYE431K with p2HGPDER/CYC and pUCΔSS-ERE
AFYS485YER	AFYS485Y with p2HGPDER/CYC and pUCΔSS-ERE
AFYT525IER	AFYT525I with pJR3 and pUCΔSS-ERE
AFYG313NER	AFYG313N with p2HGPDER/CYC and pUCΔSS-ERE
MYY405	**a** *leu2 his3 URA :: YDJ1*
MYY290	**a** *leu2 his3 ura3*
AFY100	MYY405 *ura3*
MYY290AR	MYY290 with pARH and pPGKareLacZC
AFY100AR	AFY100 with pARH and pPGKareLacZC
AFY100H34Q	AFY100AR with pYQPD
AFYSIS1	AFY100 with pCB338, pJR4 and pARH
AFY100-151	AFY100AR with pAV-151
AFY100-HDJ2	AFY100AR with pGALHDJ2
AFY100-HSJ1	AFY100AR with pGALHSJ1
AFYHDJ1	AFY100 with pARU, pYX233hsp40 and pJR4
AFYgalYDJ1	AFY100 with pGALYDJ1, pARH and pJR4
AFY34Q/SIS	AFY100 with pYQPD, pARH and pCB338
AFYpAV7	AFY100AR with pAV7
ACY95cFOA	**a** *ade2 leu2 his3 trp1 ura3 can1 ydj1 :: HIS3*
ACY95ER	**a** *ade2 leu2 his3 trp1 ura3 can1 ydj1 :: HIS3* with pJR3 and pUCΔSS-ERE
ACY95pAV7	**a** *ade2 leu2 his3 trp1 ura3 can1 ydj1 :: HIS3* with pJR3, pUCΔSS-ERE and pAV7
W3031ER	W3031a with pJR3 and pUCΔSS-ERE
8A7	α *cdc37-34, leu2, lys2, trp1, ura3*
AFY14	8A7 with pPGKareLacZC and pG1-hAR
AFY15	AFY14 with pRS315
AFY16	AFY14 with pRS315CDC37

AFY17	α *CDC37* :: *cdc37-34, leu2, lys2, trp1, ura3* with pPGKareLacZC and pG1-hAR
AFY18	AFY14 with pGALCDC37
AFY14-GR	8A7 with pPGKareLacZC and pGN795
AFY17-GR	α *CDC37* :: *cdc37-34, leu2, lys2, trp1, ura3* with pPGKareLacZC and pGN795
AFY14-ER	8A7 with pJR3 and pUCΔSS-ERE
AFY16-ER	α *cdc37-34, leu2, lys2, trp1, ura3* with pRS315CDC37, pJR3 and pUCΔSS-ERE
AFY17-ER	α *CDC37* :: *cdc37-34, leu2, lys2, trp1, ura3* with pJR3 and pUCΔSS-ERE
AFY14ABC	α *cdc37-34, leu2, lys2, trp1, ura3* pABC and pPGKareLacZC
AFY17ABC	α *CDC37* :: *cdc37-34, leu2, lys2, trp1, ura3* with pABC and pPGKareLacZC
AFY14GAL	α *cdc37-34, leu2, lys2, trp1, ura3* with pPGKgal-hAR
AFY17GAL	α *CDC37* :: *cdc37-34, leu2, lys2, trp1, ura3* with pPGKgal-hAR

CHAPTER 5

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IMAGE EVALUATION
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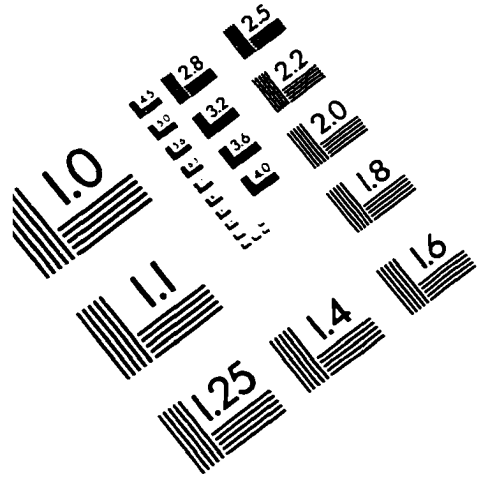

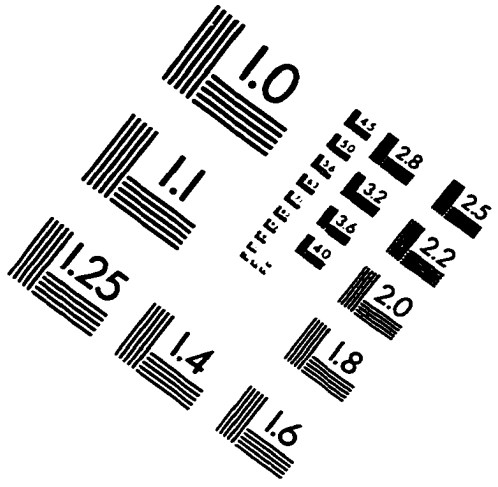

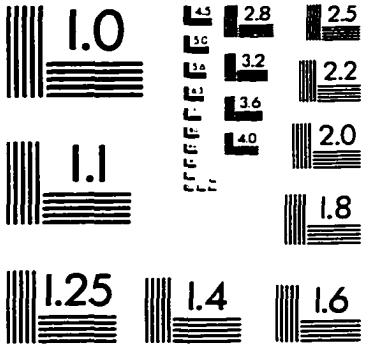

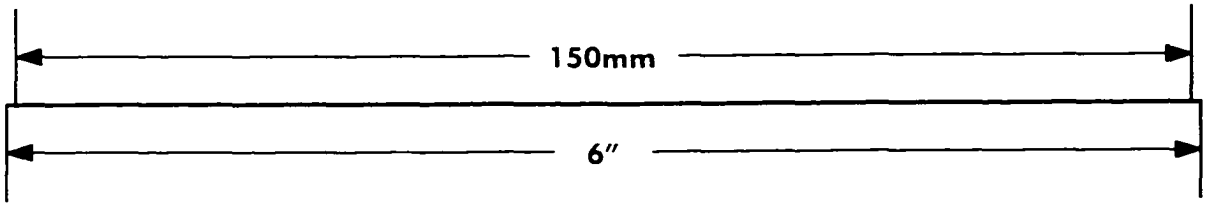

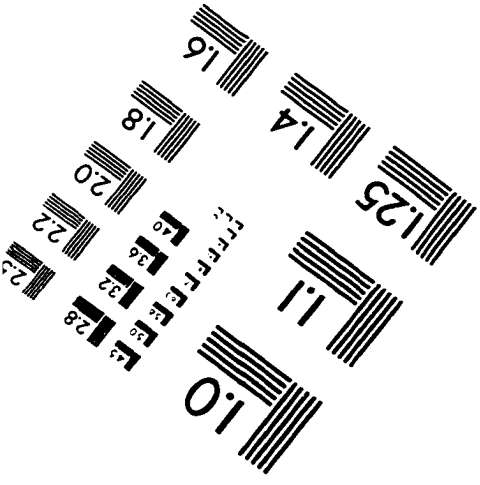

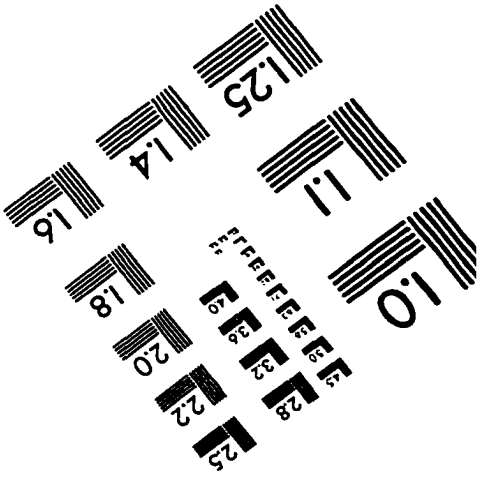

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