CHARACTERIZATION OF p50^{cdc37}, THE KINASE-SPECIFIC COMPONENT OF THE HSP90 CHAPERONE MACHINERY

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PREFACE

Molecular chaperones are proteins that assist the assembly of other proteins and protein oligomers by binding to hydrophobic regions that are transiently exposed during various cellular processes thereby inhibiting nonproductive interactions that may otherwise produce non-functional structures. Hsp90 has been termed the "signal transduction chaperone" based on its interactions with steroid hormone receptors and tyrosine kinases involved in signal transduction pathways. Steroid hormone receptors require hsp90 chaperone machinery to maintain a conformation capable of hormone binding while some tyrosine kinases require hsp90 for maturation. CDC37 is a widely expressed protein that participates in cell processes controlled by kinases. CDC37 homologues are found in yeast, Drosophila, and mammalian cells. Studies in mammalian cells have found a CDC37 homologue protein, $p50^{cdc37}$ to be important in kinase activity, specifically $pp60^{v\text{-}src}$, CDK4, and raf. The role of $p50^{cdc37}$ and hsp90 in kinase maturation is not certain and is complicated by the fact that hsp90 can bind to $p50^{cdc37}$ in the absence of a kinase. In my thesis, I attempted to illuminate the roles and mechanisms underlying $p50^{cdc37}$ function by analyzing its interaction with hsp90. The first step in this process is to determine the stability of the $p50^{cdc37}$/hsp90 complex in the absence of a kinase. Treatment of the $p50^{cdc37}$/hsp90 complex with geldanamycin and molybdate would help determine how $p50^{cdc37}$ interacts with hsp90. The final step in understanding the interaction between $p50^{cdc37}$ and hsp90 is to determine if $p50^{cdc37}$ acts in concert with

hsp90 cohorts as part of the hsp90 chaperone machinery. My data allow me to draw three significant conclusions. The first is that $p50^{cdc37}$ and hsp90 are weakly associated in the absence of a kinase. Additionally, the interaction between $p50^{cdc37}$ and hsp90 is not dependent on phosphorylation events nor does ATP binding to hsp90 effect the interaction. I have also shown that $p50^{cdc37}$ is found in hsp90 complexes along with other hsp90 cohorts.

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NOMENCLATURE

ATP	adenosine triphosphate
Cyp40	Cyclosporin A binding protein 40
DTT	Di-thiothreitol; used to denature protiens for SDS-PAGE
FKBP52	FK506 binding protein 52
hsp	heat shock protein
IEF	Isoelectric focusing
IgG	immunoglobulin G
µl	microliter
p	peptide (when used in front of a number)
PT	10 mM PIPES (pH 7) plus 0.05% Tween-20
P50	10 mM PIPES (pH 7), 50 mM NaCL
P50T	10 mM PIPES (pH 7), 50 mM NaCL plus 0.05% Tween-20
P150T	10 mM PIPES (pH 7), 150 mM NaCL plus 0.05% Tween-20
P20T	10 mM PIPES (pH 7), 20 mM NaCL plus 0.05% Tween-20
P20MT	P20T with molybdate
PBS	Phosphate-buffered Saline
PR	Progesterone Receptor
PVDF	polyvinylidene difluoride
SB	Sample buffer

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	Steroid Hormone Receptor
TBS	Tris-buffered Saline
TBS/Tween	Tris-buffered Saline plus 0.05% Tween-20

CHAPTER I

INTRODUCTION

The hsp90 Chaperone Machinery

Molecular chaperones are proteins that assist the assembly of other proteins and protein oligomers by binding to hydrophobic regions that are transiently exposed during various cellular processes [1] thereby inhibiting nonproductive interactions that may otherwise produce non-functional structures. Chaperone binding and the subsequent release of the transiently exposed hydrophobic regions of the substrate protein prevent the formation of aggregates while allowing the substrate to reach a stable conformation. Some chaperones, such as hsp90, seem to be involved with specific functions within the cell. Hsp90 has been termed the "signal transduction chaperone" based on its interactions with steroid hormone receptors and tyrosine kinases involved in signal transduction pathways [2]. Steroid hormone receptors require hsp90 chaperone machinery to maintain a conformation capable of hormone binding while some tyrosine kinases require hsp90 for maturation [3].

The biochemical properties of hsp90 are consistent with its postulated role as a chaperone. Hsp90 is an abundant, ubiquitously expressed, and highly conserved protein. Hsp90 binds ATP and it has been shown that hsp90 has an ATPase activity indicating

that the hydrolysis of ATP may be important in the interaction of hsp90 with other proteins. In addition to a site for ATP binding, evidence suggests that there are two chaperone sites on hsp90 that bind substrate [4]. Evidence of two sites comes from studies using either the N-terminal domain or the C-terminal domain of hsp90 and their ability to prevent aggregation of an unfolded fragment of the steroid hormone receptor or denatured citrate synthase, a representative structured folding intermediate. These studies showed that the C-terminal site binds preferentially with partially unfolded proteins such as occurs under stress conditions and during *de novo* folding while binding to the N-terminal site appears to have more substrate specificity. Substrate binding to the C-terminal chaperone site is ATP-independent and may be regulated by hsp90's co-chaperones such as p60, hsp70, and FKBP52 which have been shown to bind to the C-terminal domain of hsp90. The N-terminal site preferentially binds unfolded polypeptides followed by dissociation after ATP binds to hsp90. These two sites may help hsp90 guide target proteins through folding processes while it is performing general chaperone functions [4].

During steroid hormone receptor (SHR) complex formation the hsp90 chaperone machinery is believed to act as a foldosome. This foldosome is a multiprotein chaperoning complex that is preassociated with hsp90 and includes all of the factors required for SHR-hsp90 heterocomplex assembly. The proteins involved include hsp90, hsp70, p60 (HOP), p48 (HIP), p23 and the immunophilins FKBP52 and Cyp-40. Heat shock protein 70 (hsp70) is an abundant general "housekeeping" molecular chaperone that binds and releases hydrophobic segments of its substrate proteins in an ATP regulated manner. As a part of the SHR complex hsp70 may provide a protein unfoldase

activity needed for hsp90 binding to the steroid hormone-binding domain of steroid receptors [5]. DnaJ (hsp40) binds to and directs substrates to hsp70. Upon substrate binding to hsp70, DnaJ stimulates hydrolysis of the ATP associated with hsp70 and dissociates from hsp70. For purified hsp70 to form an association with purified hsp90, p60 HOP needs to be present [2]. p60 HOP binds to both hsp90 and hsp70 independently in an ATP-independent manner. p60 provides a physical link between hsp90 and hsp70 and is proposed to modulate the activity of both these proteins [2]. p23 binds directly to hsp90 in an ATP-dependent manner and forms a stable complex with hsp90 and SHR in the presence of ATP. The roles of the immunophilins FKBP52 and Cyp40 during SHR complex formation are unknown.

The components of the hsp90 foldosome machinery may act as several discrete machineries [6]. Studies using purified components show that a minimal core complex of hsp70-hsp90-p60 is sufficient to convert the glucocorticoid receptor (GR) hormone binding domain from a non-steroid binding to a steroid binding conformation. This is followed by p23 binding to stabilize the resulting GR-hsp90 heterocomplex [7]. However, the other proteins found in the foldosome may be required for optimal performance *in vivo*. The exact role of the immunophilins FKBP52 and Cyp40 in SHR-hsp90 heterocomplex formation is not certain. Current evidence, based on complexes reconstituted from purified components *in vitro*, suggests that the immunophilins FKBP52 and Cyp40 are not found in the same SHR complex [8]. FKBP52 and Cyp40 both have tetratricopeptide repeat (TPR) domains. The TPRs are degenerative sequences of 34 amino acids of three tetratricopeptides repeats. The TPRs are the peptide sequences that are responsible for the binding of these proteins to hsp90. The mutual exclusivity of

FKBP52 and Cyp40 in SHR complexes may be due to a common or overlapping TPR binding sites for FKBP52 and Cyp40 on hsp90 preventing both from being bound to hsp90 at the same time [8]. This is supported by the fact that FKBP52 and Cyp40 had similar binding patterns to mutated forms of hsp90 [9].

Modeling in the cell-free rabbit reticulocyte lysate system suggests an ordered assembly pathway for formation of the steroid hormone receptor-chaperone heterocomplex. The introduction of a cell free system, rabbit reticulocyte lysate (RRL), that duplicates *in vivo* process involved with SHR formation has simplified the study of chaperone complex formation involved in SHR. Rabbit reticulocyte lysate has been used to reconstitute immunoadsorbed or DNA-bound GR into a functional GR-hsp90 complex [5] and this reconstitution seems to mimic the *in vivo* formation of SHR-hsp90 complexes. Studies using RRL have also shown that progesterone receptors (PR) free of associated proteins can be reconstituted into PR-hsp90 heterocomplexes [5]. The model of receptor-hsp90 heterocomplex assembly is based on time course studies using immunoadsorbed PR incubated in RRL. Immunoadsorbed PR is found with p60 and hsp70 immediately after incubation in RRL with peak recovery of p60 and hsp70 occurring around 4 minutes followed by a decrease as incubation continued at 30°C. p23 was shown to associate with the PR after 2 minutes of incubation then quickly reached a plateau with no decline in recovery. The initial interaction between the SHR and the chaperones occurs with the preassociated hsp90-hsp70-p60 complex. This is followed by p60 dissociation and the tight binding of p23, which acts to stabilize the complex. The dissociation of p60 is believed to vacate a TPR binding domain on hsp90 allowing an

immunophilin to bind to hsp90 leading to a final complex of SHR-hsp90-p23-immunophilin [10].

Geldanamycin and Molybdate

Two compounds, geldanamycin and molybdate alter the composition of the hsp90 chaperone machinery. Geldanamycin, a benzoquinonoid ansamycins, is a fungal anti-tumor agent that induces an accumulation of the chaperone machinery typical of intermediate-stage steroid hormone complexes. Geldanamycin increases the amount of SHR complexes containing hsp90, hsp70, and p60 with a concomitant decrease of mature complexes that contain hsp90, p23 and immunophilins [3].

Geldanamycin's effects on hsp90 have been well studied. It appears that hsp90 alternates between two binding conformations depending on the presence or absence of ATP and that switching between these alternate hsp90 conformations is required for the maturation of hsp90's target protein. It appears that geldanamycin inhibits this switching [11]. Based on crystallography data of the hsp90 N-terminal domain crystallized with geldanamycin, geldanamycin interacts with hsp90 in a manner similar to ADP [12] at an ATP binding site and it is proposed that geldanamycin locks hsp90 into its ADP-bound conformation [13]. The consequences of geldanamycin binding to hsp90 include the disruption of hsp90-dependent signaling proteins in mammalian cell cultures and disruption of function of hsp90 target proteins expressed in yeast [14]. Treatment with geldanamycin SHRs in an intermediate phase of formation leading to the subsequent degradation of the SHR [11]. It has also been shown that by binding to hsp90,

geldanamycin inhibits the formation of $p50^{cdc37}$/hsp90/$pp60^{v\text{-}src}$ complexes, resulting in the loss of tyrosine kinase activity [15].

Molybdate enforces an alternative conformation of hsp90. Molybdate stabilizes steroid receptor function *in vivo*, the receptor hsp90 interaction, and increases the presence of "late" hsp90 cohorts. Immunoadsorptions of molybdate treated receptor complexes yield higher amounts of p23 and immunophilins than untreated receptors. This effect indicates that it is the late phase of SHR complex formation that is affected. Molybdate stabilizes the interactions of hsp90 with other proteins, including kinases; therefore hsp90 seems to be the target of the stabilizing effect rather than the SHR. One possible mechanism is that by being a transition metal oxyanion, molybdate resembles the transition state of phosphate hydrolysis and therefore interacts with the phosphate-binding sites of proteins. If this is the case with hsp90, molybdate might interact with the ATP binding site and thereby stabilize this conformation [10]. It is thought that the final step of SHR activation is binding of p23 followed by ATP hydrolysis and the subsequent release of ADP [16]. If the ATP cannot be hydrolyzed, chaperone turnover might be prevented forcing the receptor to remain in a complex with the late phase chaperones leading to the stability of the late SHR complex conformation.

Studies using the nucleotide-binding pocket of DnaK/hsp70 chaperones show that ADP binding keeps them in a "closed" or "slow-on, slow-off" conformation. Conversely, ATP binding changes them to an "open" or "fast-on, fast-off" conformation allowing the folding substrate to fold, if it can, while loosely bound, or remain in the chaperone complex [15]. Studies in hsp90 show that molybdate or geldanamycin treatment locks hsp90 in either a high or low affinity conformation respectively and stops

the hsp90-mediated processes of biogenesis and renaturation suggesting that neither mode is sufficient for proper hsp90 functioning [15].

Background of CDC37 and p50

CDC37 is a widely expressed protein that participates in cell processes controlled by kinases. CDC37 homologues are found in yeast, Drosophila, and mammalian cells. CDC37 appears to be necessary for the maturation of some kinases after transcription takes place. CDC37 was first identified in S. cerevisiae as a temperature-sensitive mutant involved in the cell division cycle arrest. Mutation of *cdc37* results in G1 phase arrest [17]. It was subsequently found that CDC37 prevents cell cycle progression because it is necessary for the activation of CDC28 a cyclin-dependent kinase involved in cell cycle control [18]. CDC37 is also associated with a yeast kinase, MPS1p, involved with spindle pole body duplication during mitosis [19]. Mutations of a CDC37 homologue in Drosophila were found to reduce signaling by the sevenless receptor. The sevenless receptor is a tyrosine kinase required for differentiation of the R7 photoreceptor neuron during embryonic development [20]. Studies in mammalian cells have found a CDC37 homologue protein, $p50^{cdc37}$ to be important in kinase activity, specifically $pp60^{v\text{-}src}$, CDK4, and raf [2].

The specific function of $p50^{cdc37}$ was not known and it was determined that genetic analysis of *cdc37* might reveal important incites about the function of $p50^{cdc37}$. The first vertebrate CDC37 gene reported was found in a cDNA library constructed from embryonic chick heart muscle mRNA. DNA analysis suggested that avian $p50^{cdc37}$ might contain glycosaminoglycan-binding motifs. The glycosaminoglycan hyaluronan binds to

proteins of some cell types and changes the way those cells proliferate, but how this occurs is not well understood [21]. However there is no evidence that $p50^{cdc37}$ binds to glycosaminoglycan *in vivo*. The chick *cdc37* gene has been analyzed and there are possible binding sites in the promoter region of *cdc37* for transcription factors that are important in gene expression regulation during tissue and organ development. If these transcription factors are active in the regulation of *cdc37* expression, $p50^{cdc37}$ may be important in cellular behavior and in morphogenesis along with its role during kinase maturation [22]. Recent studies have shown that $p50^{cdc37}$ may play a positive role in cell proliferation as evidenced by the fact that increased levels of $p50^{cdc37}$ are associated with growth-related abnormalities in mouse prostate [23]. A comparison of the $p50^{cdc37}$ sequence with other proteins revealed no significant structural homologies, suggesting that $p50^{cdc37}$ is a unique protein. This is supported by the fact that monoclonal and polyclonal antibodies against $p50^{cdc37}$ do not recognize any other protein [24].

Studies of $p50^{cdc37}$ have shown that $p50^{cdc37}$ has *in vitro* chaperone activity, which protect proteins from aggregation. It was suggested that $p50^{cdc37}$ might regulate the mammalian cell cycle through a direct affect on CDK4, a mammalian cyclin-dependent kinase, by facilitating the proper folding for CDK4 assembly with the cyclins [25]. As chaperones, $p50^{cdc37}$and hsp90 prefer different target proteins and have different effects [26]. $p50^{cdc37}$ appears to be a protein kinase targeting subunit of the hsp90 chaperone machinery. The absence of $p50^{cdc37}$ in insect cells expressing GST-cdk4 prevents the formation of a cdk4 complex that contains hsp90, yet when $p50^{cdc37}$ is co-expressed hsp90 is found complexed with cdk4. The $p50^{cdc37}$/hsp90 complex binds preferentially to inactive CDK4 that is not bound to cyclins. Inactivation of $p50^{cdc37}$ decreases the half-life

of newly synthesized CDK4. The p50^{cdc37}/hsp90 complex might stabilize CDK4, suggesting a chaperone-dependent step in CDK4/cyclin D complex formation [27].

Perhaps the best characterized interaction of p50^{cdc37} and hsp90 with a protein is the interaction of these proteins with the Rous sarcoma virus (RSV) encoded protein viral-pp60^{src} [28]. RSV is an oncogenic retrovirus whose transforming properties come from a protein tyrosine kinase called pp60$^{v\text{-}src}$. Infection with RSV induces changes in morphology, metabolism, and growth control of cells that lead to the transformation of the cells into cancer cells. pp60$^{v\text{-}src}$ interacts with two cellular proteins; hsp90 and p50^{cdc37}. Hsp90 and p50^{cdc37} were found to be in a complex with pp60$^{v\text{-}src}$ by the ability of anti-pp60$^{v\text{-}src}$ antibody to co-immunoadsorb pp60$^{v\text{-}src}$ with hsp90 and p50^{cdc37} from RSV-transformed cells. The hsp90/ p50^{cdc37}/pp60$^{v\text{-}src}$ complex is stable when incubated in high salt (2M NaCl) but it dissociates in the absence of salt. Other retrovirus-encoded tyrosine kinases related to pp60$^{v\text{-}src}$ are also found complexed with hsp90 and p50^{cdc37}, suggesting a common set of events in tyrosine kinase maturation.

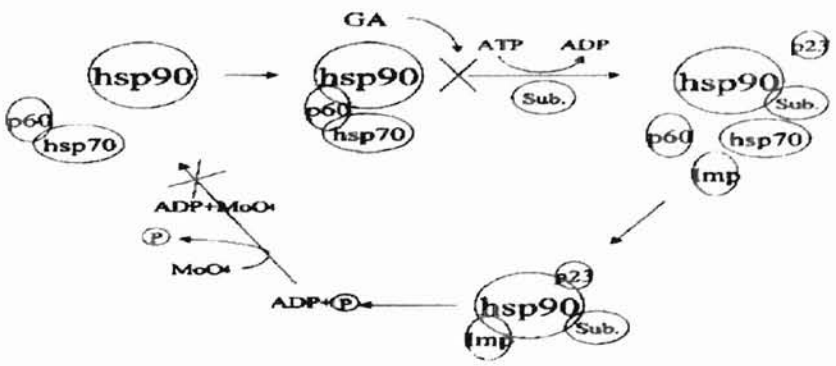
The role of p50^{cdc37} and hsp90 in kinase maturation is not certain and is complicated by the fact that hsp90 can bind to p50^{cdc37} in the absence of a kinase. Mature pp60$^{v\text{-}src}$ is found associated with the plasma membrane, however pp60$^{v\text{-}src}$ mutants that do not dissociate from p50^{cdc37} and hsp90 are not found associated with the plasma membrane. One possible role for hsp90 and p50^{cdc37} is that they may transport or direct newly synthesized pp60$^{v\text{-}src}$ to the plasma membrane. Another possibility is that p50^{cdc37} and hsp90 might facilitate transport of pp60$^{v\text{-}src}$ to the membrane indirectly, by keeping pp60$^{v\text{-}src}$ in a soluble form until it reaches the membrane. However, soluble pp60$^{v\text{-}src}$ not complexed with p50^{cdc37} and hsp90 has been detected, although it may have been

dissociated from the membrane during the homogenization of the cell. Never the less, it has not been conclusively demonstrated that hsp90 and $p50^{cdc37}$ are essential for maintaining $pp60^{v\text{-}src}$ solubility. In a resent study [29] it was found that the $p50^{cdc37}$/hsp90/kinase complex is essential for the proper function of Raf-1, a component of the MAPK phosphorylation cascade. It was found that a deletion mutant $p50^{cdc37}$ that could not bind to hsp90 inhibited Raf-1 and MAPK activation, and geldanamycin treatment prevented complex formation and Raf-1 kinase activation in serum [29]. This represents further evidence that the $p50^{cdc37}$/hsp90 complex is necessary for tyrosine kinase activation.

$p50^{cdc37}$ is a phosphoprotein and phosphorylation may play a role in its regulation. Analysis of $p50^{cdc37}$ adsorbed with hsp90 and $pp60^{v\text{-}src}$ from chicken cells indicates that $p50^{cdc37}$ associated with $pp60^{v\text{-}src}$ contains both phosphoserine and phosphotyrosine whereas $p50^{cdc37}$ from uninfected cells only has a phosphoserine. It has been suggested that $pp60^{v\text{-}src}$ might directly phosphorylate $p50^{cdc37}$, however tyrosine phosphorylation of $p50^{cdc37}$ may not be crucial for hsp90/ $p50^{cdc37}$/ $pp60^{v\text{-}src}$ interactions. This is based on studies of $pp60^{v\text{-}src}$ transformed rat or mouse cells and temperature sensitive $pp60^{v\text{-}src}$ viruses. $p50^{cdc37}$ is not phosphorylated on tyrosine in rat or mouse cells which otherwise show the typical transformed phenotype. Cells infected with viruses encoding a temperature sensitive *src* gene were found to contain phosphorylated $p50^{cdc37}$. It was also found that that $p50^{cdc37}$ was not phosphorylated in all transformed cells [28]. Therefore, it appears that $p50^{cdc37}$ phosphorylation is not essential for cellular transformation or for the association and dissociation of $pp60^{v\text{-}src}$ with hsp90 and $p50^{cdc37}$.

It is clear that more information about the interaction between p50^{cdc37} and hsp90 is needed to understand how they are involved with tyrosine kinase maturation. In my thesis, I attempted to illuminate the roles and mechanisms underlying p50^{cdc37} function by analyzing its interaction with hsp90. The first step in this process is to determine the stability of the p50^{cdc37}/hsp90 complex in the absence of a kinase. To determine if p50^{cdc37} has other possible functions unrelated to its association with hsp90, it should be determined if all the p50^{cdc37} in a cell is in a complex with hsp90. Possible regulation of the p50^{cdc37} is an important topic due to its involvement in kinase maturation. Since p50^{cdc37} can be phosphorylated it is important to know if the interaction between p50^{cdc37} and hsp90 is dependent on, and may therefore be regulated by phosphorylation events. Treatment of the p50^{cdc37}/hsp90 complex with geldanamycin and molybdate would help determine how p50^{cdc37} interacts with hsp90. The final step in understanding the interaction between p50^{cdc37} and hsp90 is to determine if p50^{cdc37} acts in concert with hsp90 cohorts as part of the hsp90 chaperone machinery and if so how do geldanamycin and molybdate affect the interaction between p50^{cdc37} and the components of the hsp90 machinery. This project was designed to help elucidate how p50^{cdc37} interacts with hsp90 and to allow the future clarification of the role p50^{cdc37} performs in the cell.

Model of the hsp90 Machinery



CHAPTER II

MATERIALS AND METHODS

Materials

Mouse nonimmune antibody was purchased from Sigma Chemicals as Mouse IgG1,κ(MOPC-21) Sigma-M7894. Rabbit anti-hsp90 was purchased from Affinity Bioreagents (ABR) as polyclonal (Rabbit) Anti-Heat Shock Protein 86 Anitbody ABR, PA3-013. Monoclonal $p50^{cdc37}$ was purchased form ABR as Monoclonal (Mouse) Anti-Cdc37 Antibody ABR, MA3-029. Anti-cyp40 was also purchased from ABR as Polyclonal (Rabbit) Anti-Cyclophilin 40 (C-Terminal Peptide) Antibody ABR PA3-022. The anti-hsp40 antibody was purchased from StressGen as Rabbit Anti-Hsp40 Polyclonal Antibody StessGen SPA-400 along with Ec1 [30]: Anti-Hsp56 (p59) as Monoclonal (mouse)Antibody StressGen SRA-1400. Dr. David Smith of the University of Arizona kindly donated the anti-p60 (HOP) antibody F5 [31]. The anti-p23 monoclonal antibodies JJ3 and JJ5 were a gift from Dr. David Toft, Mayo Medical School [32]. Mouse polyclonal ascites anti-$p50^{cdc37}$ were prepared at Oklahoma State University. The protein molecular weight markers used were Bio-Rad's Broad Range SDS-PAGE Molecular Weight Standards, Bio-Rad 161-0317.

Methods

Immunoadsorption of Proteins

Immunoadsorptions were done by binding the anti-chaperone antibody of choice to Sepharose beads that were previously crosslinked to goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-mouse IgM antibodies. The antibodies were cross-linked to agarose resins according to the protocol previously described [33]. The antibody directed against the protein under investigation was bound to 15-20 microliters of the appropriate cross-linked anti-antibody resin by resuspending in 400 microliters of P150T. The tube with resin and antibody were rocked for 1 hour at 4°C and unbound material was washed off with three 1ml washes of P50. After the final wash all buffer was aspirated to minimize dilution of the lysate mixture to be adsorbed. Lysate mixtures were typically adsorbed for 2 hours on ice with stirring. The unbound proteins were washed off as indicated in the figure ledgends. Bound proteins were eluted with SDS-PAGE sample buffer, separated on SDS-PAGE gels and analyzed by western Blotting.

Preparation of Lysate

Rabbit reticylocyte lysate reaction mixtures or protein synthesis (PS) mixes contained 50% (v/v) rabbit reticylocyte lysate, an ATP-regenerating system (10 mM creatine phosphate and 20 units/ml creatine phosphokinase), and other components as previously described [15]. The PS mixes used contained 20 μM hemin unless otherwise indicated. Incubation times and temperatures varied and are specified in the figure legends. To remove any aggregates of denatured proteins, all PS mixes were chilled on ice for 5 min then clarified at full speed in the Eppendorf horizontal centrifuge at 4°C.

When aliquoting the lysate to the resin, care was taken to avoid the pellet at the bottom of the clarified lysate, excess lysate was prepared to facilitate this.

Western Blotting

Proteins were denatured with reducing SDS-PAGE sample buffer, separated by SDS-PAGE and electro-transferred to PVDF membranes (Bio-Rad) for analysis. The membranes were blocked in TBS/5% skim milk for one hour at room temperature. Primary antibodies were added at the concentration determined to be optimal for the particular antibody and bound overnight at 4°C. The blots were washed 1x TBS, 2xTBS/Tween-20, and 1xTBS/5% skim milk 5 minutes each. Secondary antibodies conjugated with alkaline phosphatase were then used to detect the bound primary antibodies for two hours at room temperature. Blots were washed 1x TBS 2xTBS Tween-20 and 2xTBS 5 minutes each. Blots were incubated in an alkaline phosphatase reaction buffer containing NBT and BCIP at 37°C for 1 to 5 min.

Plasmid Preparation

Standard molecular biology cloning techniques were used to create the plasmid containing a histidine-tagged $p50^{cdc37}$ coding sequence. The plasmid was constructed by sub-cloning two pieces of DNA from two previously made plasmid clones: (i) a 1.3 kb DNA fragment coding sequence for a six-histidine tag from Matts's clone library AC#121, and (ii) a 2.9 DNA fragment containg the $p50^{cdc37}$-coding region from Matts's clone library AC# 149. Plasmid DNA from these two clones were cut with the endonucleases NcoI and ScaI to make fragments with compatible ends.

Proteolytic Digestion of $p50^{cdc37}$

Histidine-tagged $p50^{cdc37}$ was synthesized and radiolabeled with [^{35}S] Met by coupled transcription and translation for 1 hr in nuclease-treated rabbit reticulocyte lysate (*in vitro* translation) according to standard laboratory protocols [34]. Reaction mixtures were then chilled on ice for 5 minutes then diluted 1:1 with PAB buffer. At 30 second intervals the chilled, diluted translation mixtures were added to trypsin proteolysis mixtures while on ice. After a 6-minute digestion the reactions were stopped by adding hot SDS-PAGE sample buffer followed immediately by boiling. The digestion products were analyzed by SDS-PAGE on a 10% gel and western blotting with mouse anti-histidine tag or monoclonal $p50^{cdc37}$ antibody purchased from ABR.

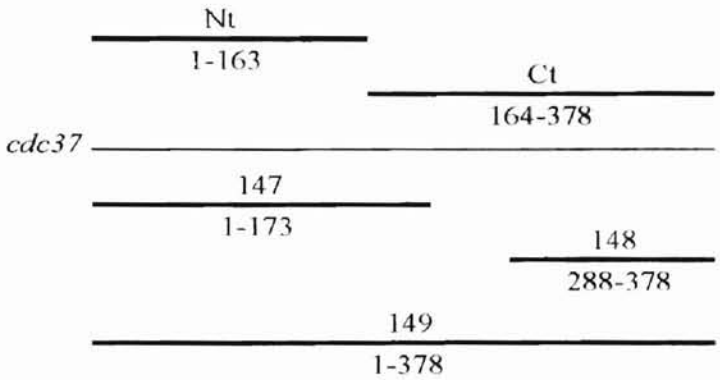
Slab Isoelectric Focusing

Vertical isoelectric focusing (IEF) was done following laboratory protocols [35] based on a protocol in [36]. Three percent acrylamide gels 1.5 mm thick were polymerized with Pharmalytes to generate a pH gradient of 4.5-6 and ran in a Bio-Rad Mini Protean II electrophoresis unit. Protein synthesis mixtures were immunoadsorbed with anti-hsp90 (8D3) antibodies. The resulting supernatants were removed by adding 60 µl of 10 mM PIPES (pH=7) plus 5 mM NaF to the immunopellets. The supernatants were centrifuged to pellet any remaining resin and 50 µl was removed to a new tube and an equivalent volume of 2x IEF sample buffer and excess urea was added. The samples were loaded into 13 mm wells and overlay buffer was used to cover the samples and to fill the empty lanes. Samples were focused at 25 volts per gel overnight. The final

focusing phase was carried out by increasing the voltage to 150 volts per gel for 2 hours. The proteins were then transferred to a PVDF membrane by wet transfer in the Genie Eletrophoretic Blotter at a constant voltage of 24 volts for 2 hours. The blots were then western blotted with anti-p50^{cdc37} antibodies.

Domain Interactions

Plasmids containing DNA fragments of the p50^{cdc37} coding sequence were translated and radiolabeled with [^{35}S] Met *in vitro* in TnT RRL. The sequences translated included plasmid encoding full-length p50^{cdc37}, the N-terminal domain (amino acids 1-163), the C-terminal domain (amino acids 164-378), and two fragments of p50^{cdc37} from amino acids 1-173 and 288-378. Immunoadsorption with anti-hsp90 antibody, 8D3, or nonimmune mIgM was performed using the translation mixes. Samples were analyzed by SDS-PAGE and western blots were performed to access if equal amounts of hsp90 were adsorbed in each lane and autoradiography performed to see which fragments of p50^{cdc37} bound to hsp90.



CHAPTER III

RESULTS

The $p50^{cdc37}$/hsp90 Interaction

Characterization of the $p50^{cdc37}$/hsp90 Interaction

Previous work has demonstrated that the interaction between hsp90, $p50^{cdc37}$, and viral $p60^{src}$ is stable when exposed to high salt conditions; in fact this complex dissociates in the absence of salt [28]. To determine if the hsp90/$p50^{cdc37}$ interaction behaves differently when a kinase is not in the hsp90- $p50^{cdc37}$ complex, anti-$p50^{cdc37}$ immunoadsorptions were washed with buffers containing 50 mM to 1000 mM NaCl. Additionally, phosphate buffered saline (150 mM NaCl) was used to assess if the addition of phosphate had any significant effect on the complex because hsp90/immunophilin complexes are destabilized when exposed to phosphate in the buffer. In the presence of 50 mM NaCl, the interaction was readily detected (Figure 1, lane 3). However, in 150 mM NaCl, less hsp90 was recovered (lane 5). Additionally, the hsp90 interaction with $p50^{cdc37}$ was virtually undetectable after 500 mM and 1000 mM washes (lane 7 and 9 respectively). Phosphate buffered saline (PBS; 150 mM NaCl) had a negative effect on the recovery of hsp90 (lane 11) relative to the PIPES wash with 150 mM of NaCl (lane 5). This data shows that the interaction between hsp90 and $p50^{cdc37}$ without a kinase present is unstable. Molybdate stabilizes some proteins' interactions with hsp90, therefore I examined if molybdate stabilizes the interaction between $p50^{cdc37}$ and hsp90 in

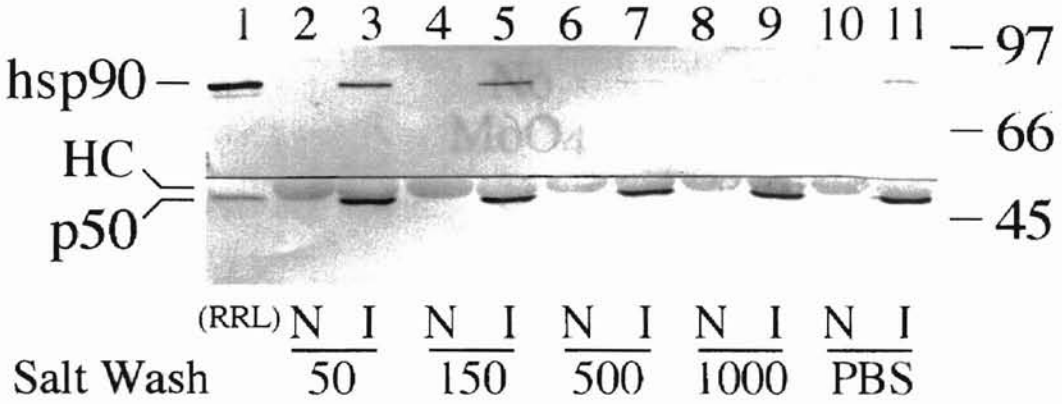


Figure 1. The stability of the p50^{cdc37} /hsp90 association in buffers of various ionic strengths.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were immunoadsorbed with nonimmune (N; lanes 2, 4, 6, 8, and 10) or anti p50^{cdc37} (I; lanes 3, 5, 7, 9, and 11) antibody. After binding, immunoadsorptions were washed with 10 mM PIPES (pH=7.0) containing 50-1000 mM NaCl as indicated or with phosphate-buffered saline (lanes 10 & 11). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*) and p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard for the detection of hsp90 and p50^{cdc37}.

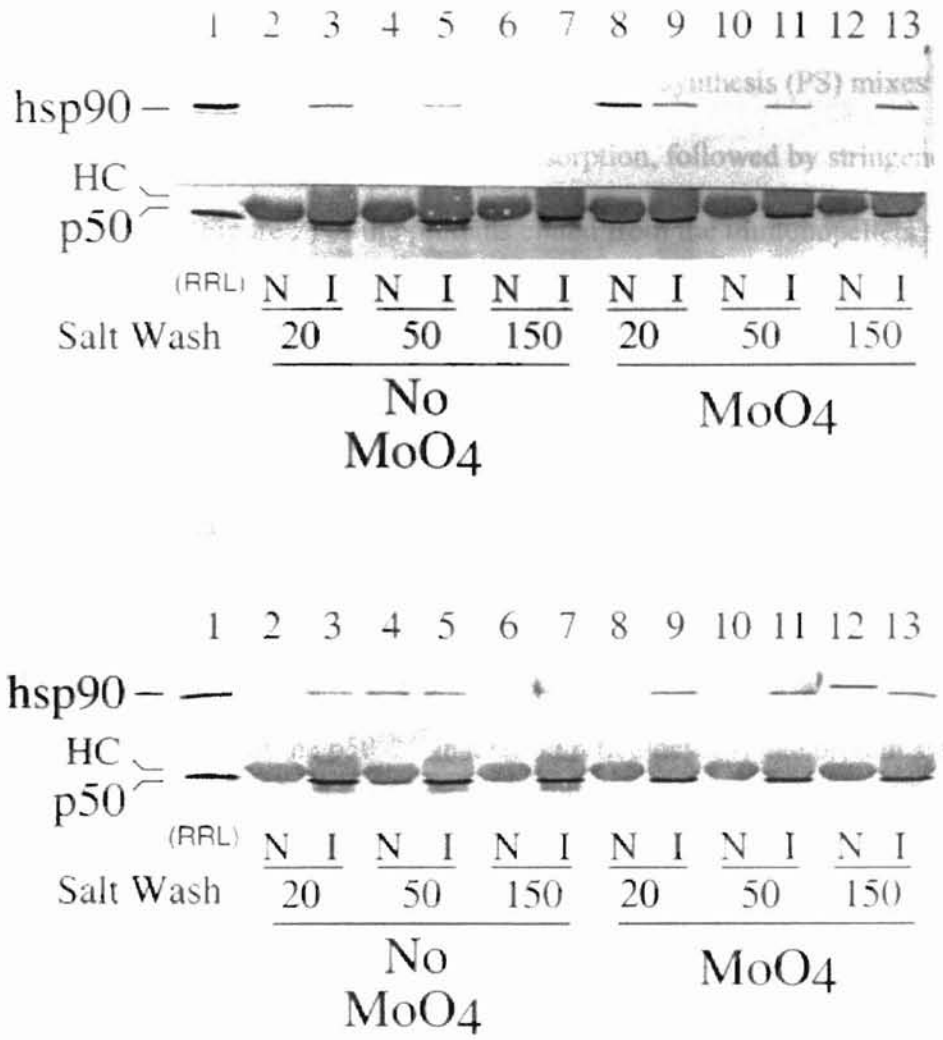


Figure 2. The stability of the p50^{cdc37} /hsp90 association in buffers of various ionic strengths after treatment with molybdate.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were treated with molybdate or water then immunoadsorbed with nonimmune (N; lanes 2, 4, 6, 8, 10 and 12) or anti p50^{cdc37} (I; lanes 3, 5, 7, 9, 11 and 13) antibody. After binding, immunoadsorptions were washed with PT containing 50-150 mM NaCl or containing 50-150 mM NaCl plus 20mM molybdate as indicated. Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*) and p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard for the detection of hsp90 and p50^{cdc37}.

side-by-side immunoadsorption experiments using protein synthesis (PS) mixes treated with molybdate and untreated PS mixes. After adsorption, followed by stringency washes, western blots (Figure 2) of the proteins eluted from the immunopellets showed that there was no decrease of detection of hsp90 in the 150 mM wash in the molybdate-treated samples (lane13). However, controls indicated a decrease in the hsp90 detection with a 150 mM NaCl wash (lane 7). These preliminary results indicate that molybdate may moderately stabilize the interaction of $p50^{cdc37}$ and hsp90, however higher stringency washes are required to determine how effective molybdate is at stabilizing this interaction.

To determine if all of the $p50^{cdc37}$ in a rabbit reticulocyte lysate protein synthesis (RRL PS) mix is found complexed with hsp90, hsp90 was quantitatively depleted from RRL PS mix by immunoadsorption with the anti-hsp90 monoclonal antibody 8D3. The unbound protein fraction was recovered and assessed to determine if all $p50^{cdc37}$ would be concomitantly depleted with hsp90. Western blot analysis (Figure 3) revealed that a significant amount of $p50^{cdc37}$ was detected in the unbound fraction, indicating that not all $p50^{cdc37}$ molecules are part of the $p50^{cdc37}$/hsp90 interaction.

Test for Possible Regulation of Complex Formation Between $p50^{cdc37}$ and hsp90

Many protein/protein interactions are regulated by the phosphorylation or dephosphorylation of one or more of the proteins involved. One class of enzymes, the phosphoprotein phosphatases, cleaves phosphate from proteins. Alkaline phosphatase

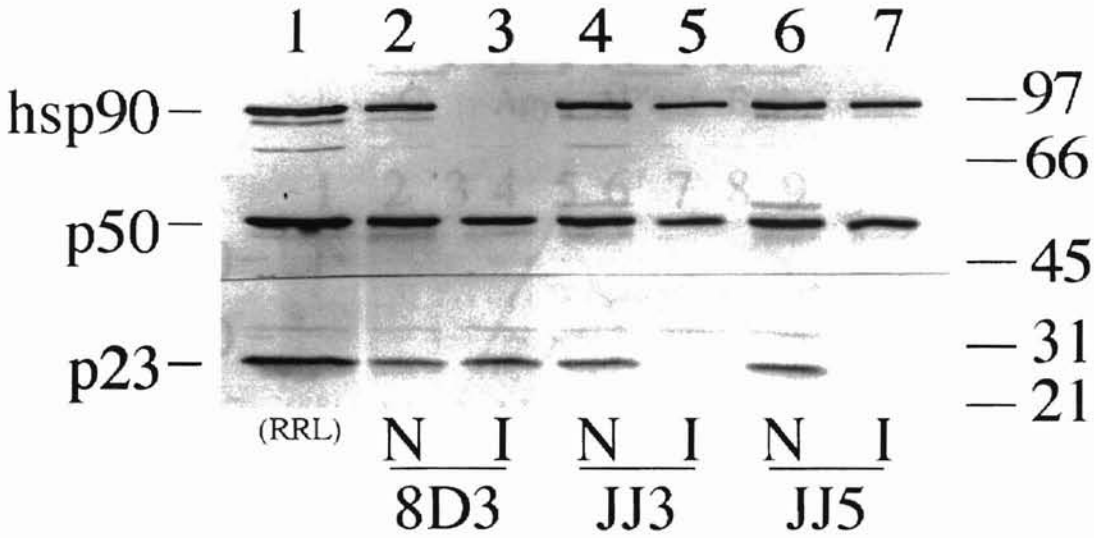


Figure 3. Depletion of Rabbit Reticulocyte Lysate of hsp90 or p23

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated 10 min at 30°C. Four micrometers of the mixture were immunoadsorbed using nonimmune antibodies (N; lanes 2, 4, & 6), anti-hsp90 *(8D3)* (I; lane 3), anti-p23 *(JJ3)* (I; lane 5), or anti-p23 *(JJ5)* (I; lane 7) antibodies to deplete either hsp90 or p23. The unbound materials were removed by washing the pellet with 160 µl of 10 mM PIPES (pH=7). 140 µl of the supernatant was added to 50 µl of 4X SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), $p50^{cdc37}$ (*p50*), and p23 *(p23)* are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

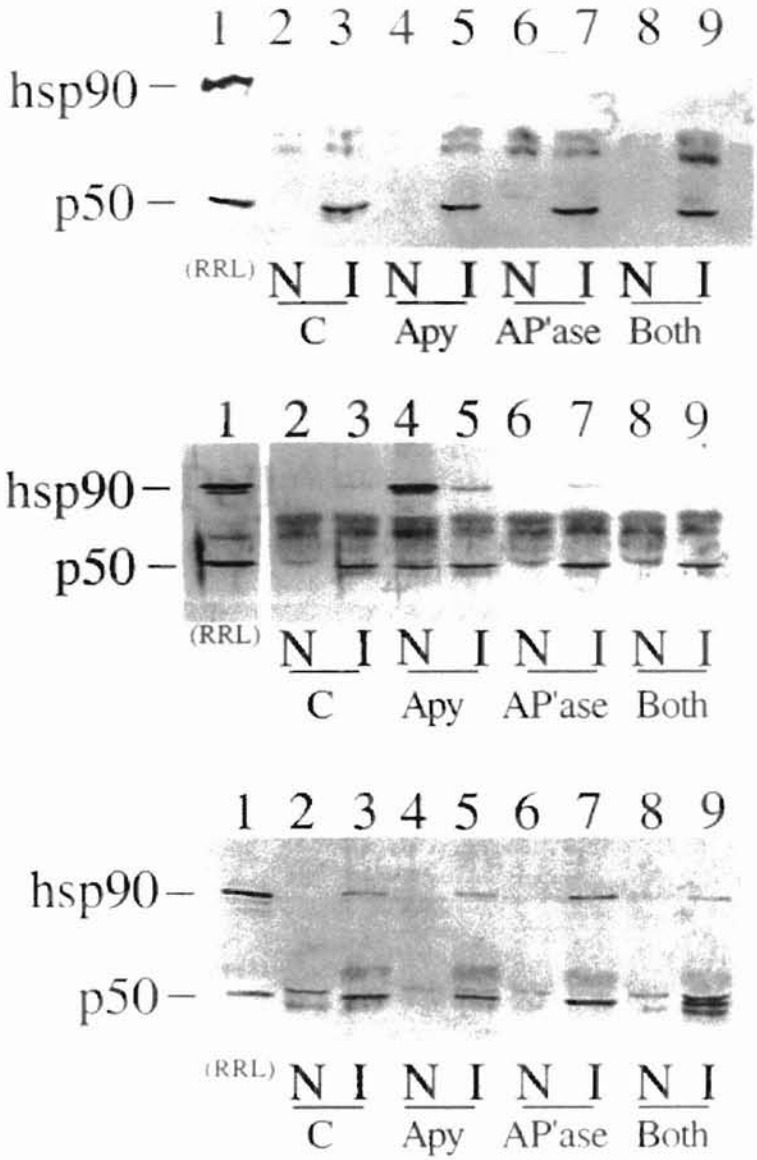


Figure 4. Effects of apyrase and alkaline phosphatase on the physical association between p50^{cdc37} and hsp90.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min apyrase (Apy; lanes 4&5), alkaline phosphatase (AP'ase; lanes 6&7), both (lanes 8&9) or water (C; lanes 2&3) were added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, 6 and 8) or with anti-p50^{cdc37} (I; lanes 3, 5, 7 and 9) After binding, the immunoadsorptions were washed four times with P50T. Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-p50^{cdc37} and anti-hsp90 antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

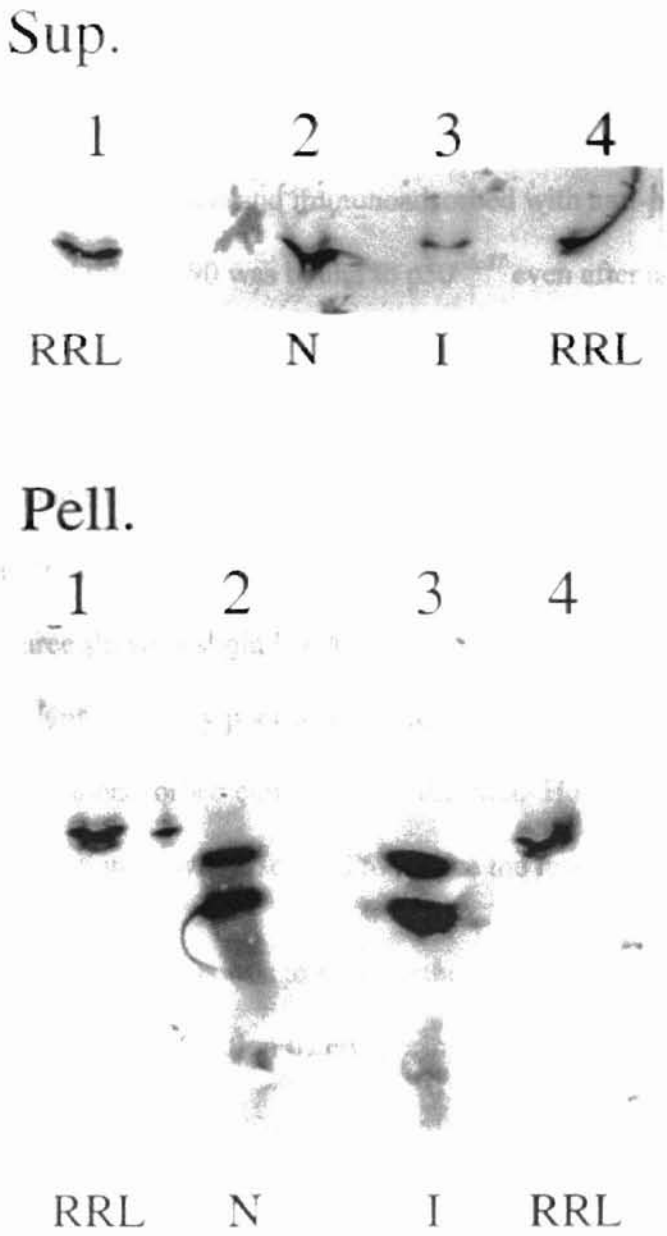


Figure 5. Depletion of Rabbit Reticulocyte Lysate of hsp90 followed by Vertical Slab-IEF.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated 10 min at 30°C. 8 of the mixture were immunoadsorbed using nonimmune antibody (N; lane 2) or anti-hsp90 *(8D3)* (I; lane 3) to deplete hsp90. The unbound materials were removed by washing the pellet twice with 30 µl of 10 mM PIPES (pH=7) plus 5 mM NaF. The supernatants were pooled and 60µl of 2x IEF SB + urea added. The samples were isoelectric focused at a pH range of 4.6-6 followed by western blotting with anti-$p50^{cdc37}$ antibodies. Immunoreactive bands representing $p50^{cdc37}$ (*p50*) are indicated. Lanes 1&4 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

was used to test if dephosphorylation of hsp90 or p50^{cdc37} altered their interaction. PS mixes were treated with phosphatase and immunoadsorbed with anti-p50^{cdc37} antibodies. Western blotting showed that hsp90 was bound to p50^{cdc37} even after alkaline phosphatase treatment (Figure 4: lane 7). Potential rephosphorylation of alkaline phosphatase treated proteins was prevented by the depletion of ATP via apyrase treatment (Figure 4: lane 9). Apyrase alone was used as a control to determine if altering the nucleotide levels effected the interaction between hsp90 and p50^{cdc37} (Figure 4: lane 5). Repeat number three shows a slight hsp90 increase in the alkaline phosphatase treated lane (lane 7). Unfortunately poor hsp90 detection and poor reproducibility in the amount of p50^{cdc37} immunoadsorbed compromised the data. However, treatment with both alkaline phosphatase and apyrase seemed to reduce the recovery of hsp90.

The next step in this process was to analyze the phosphorylation states of free p50^{cdc37} and the hsp90-bound population by comparing the immunodepleted supernatant and the immunopellet by isoelectric focusing. Unfortunately the pellet samples were unreadable, presumably by interference of the immunoadsorbing antibody (Figure 5). However, the supernatant samples could be focused. In preliminary IEF experiments there did not seem to be any distinguishable differences between the subtracted and unsubtracted lanes (Figure 5), but as can be seen in this figure the bands are not well focused. I came to realize that the seal between the two buffers was not tight and that there was mixing of the two buffers that led to poor or no focusing. The results of alkaline phosphatase treatment and isoelectric focusing of p50^{cdc37} suggest that it is

unlikely the interaction between hsp90 and p50^{cdc37} is regulated by dephosphorylation events.

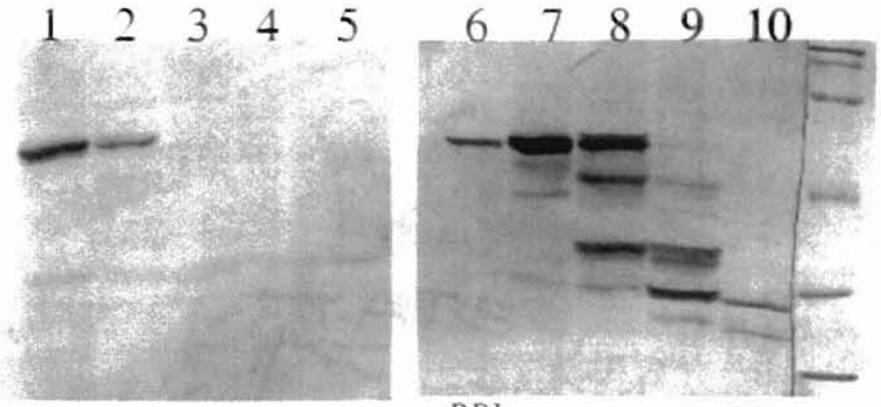
Preliminary data of p50 structure

To gather preliminary data on p50^{cdc37} domain organization, histidine-tagged p50^{cdc37} was synthesized and radiolabled with [^{35}S] Methionine via *in vitro* translation and subjected to trypsin nicking *in situ*. Half of the total volume of the nicking mixture was Western blotted with anti-histidine-tag, which would detect the N-terminus of p50^{cdc37}, and the other half was blotted with a commercial monoclonal p50^{cdc37} antibody whose epitope was unknown. The two different antibodies were used in hopes that they would show different banding patterns that could decipher the fingerprint. However, the banding patterns of both antibodies were the same (Figure 6). Autoradiographs were done to determine if there were any bands that did not appear in the western blots (Figure 6). The autoradiographs showed the same banding patterns as the western blots. Because there were no ^{35}S bands not detected by the antibody specific for the N-terminus of p50^{cdc37}, there does not seem to be any large cleavage product containing the C-terminal domain of p50^{cdc37}. This could mean that a bound protein protects the C-terminus. However proteolysis of salt-treated reaction mixtures versus low salt reaction mixtures did not show any significant differences (Figure 7).

Figure 6. Proteolytic nicking of p50^{cdc37}

p50^{cdc37} was *in vitro* translated with concomitant ^{35}S radiolabeling. The reaction mixtures were incubated for 1 hour at 30°C. The resulting translations were diluted 1:1 with PAB buffer. Proteolysis mixtures were prepped by adding 0, 0.8, 5 and 30 µl of 2x Trypsin stock to PAB buffer to make dilutions with 30 µl final volume. 30 µl of the chilled diluted translation reactions were added to the proteolysis mixtures at 30-second intervals, vortexed and placed on ice for 6 minutes. Proteolysis reactions were stopped by immediate boiling in hot 60 µl of SDS-PAGE SB. Samples were ran on 10% SDS-PAGE gels followed by autoradiography. Analysis by western blotting was preformed with anti-histidine-tag (His) and commercially available monoclonal anti-p50^{cdc37} (M p50^{cdc37}) antibodies. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 6 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

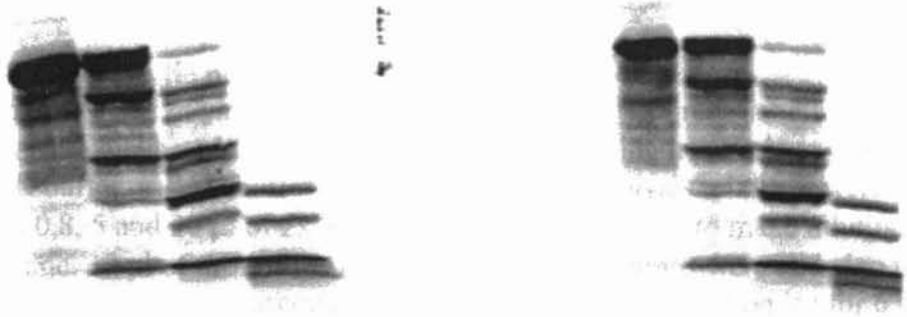
Western Blot



His RRL M p50*cdc37*

AR

His M p50*cdc37*



His M p50*cdc37*

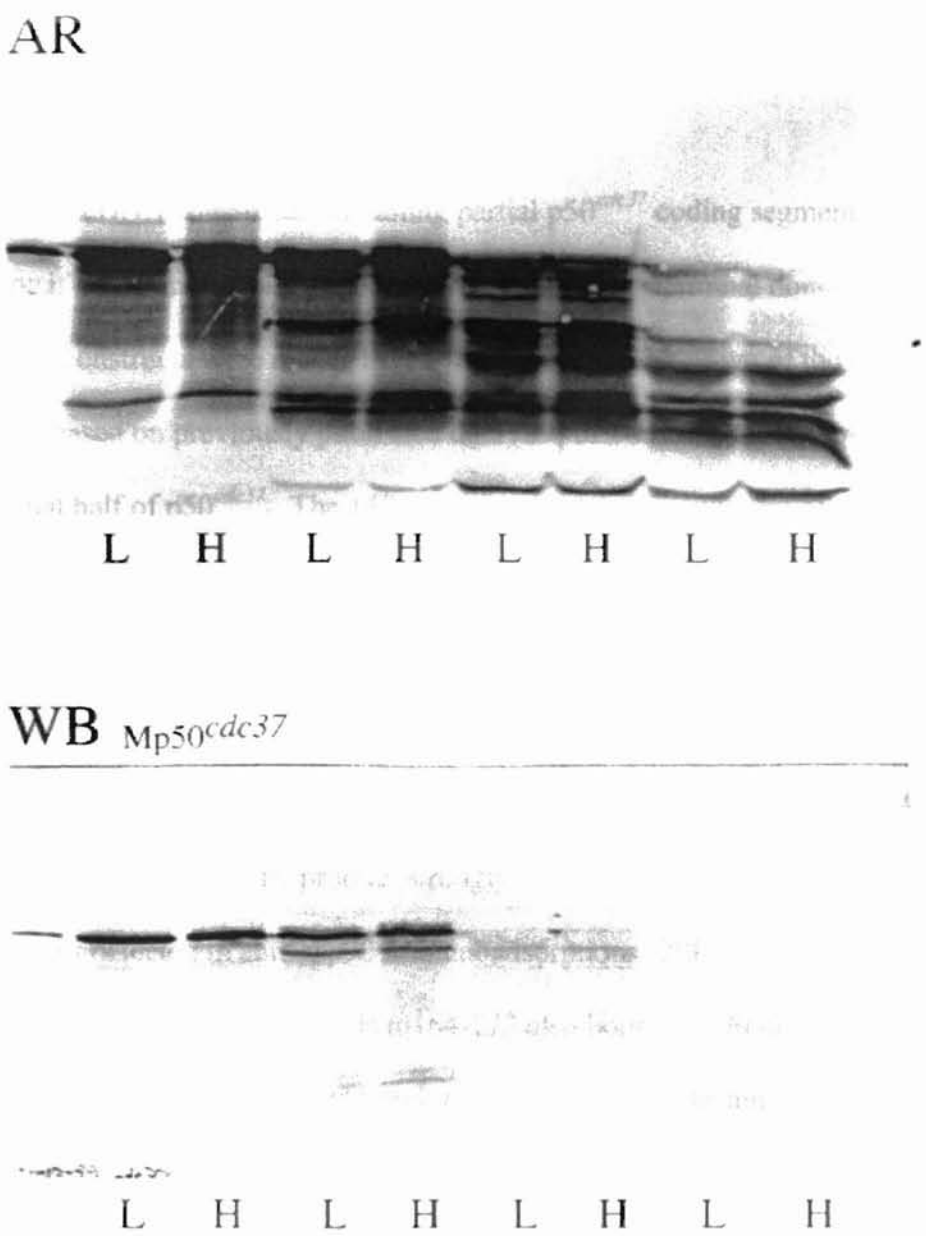


Figure 7. Proteolytic nicking of salt-treated p50^{cdc37}

p50^{cdc37} was *in vitro* translated with concomitant ^{35}S radiolabeling. The reaction mixtures were incubated for 1 hour at 30°C. The resulting translations were diluted 1:1 with PAB buffer or PAB buffer + 300mM NaCl. Proteolysis mixtures were prepped by adding 0, 0.8, 5 and 30 µl of 2x Trypsin stock to PAB buffer to make dilutions with 30 µl final volume. 30 µl of the chilled diluted translation reactions were added to the proteolysis mixtures at 30-second intervals, vortexed and placed on ice for 6 minutes. Proteolysis reactions were stopped by immediate boiling in hot 60 µl of SDS-PAGE SB. Samples were ran on 10% SDS-PAGE gels followed by autoradiography.

p50^{cdc37} Domain Interactions with hsp90

p50^{cdc37} cDNA constructs containing partial p50^{cdc37} coding segments were used to determine if hsp90 interacts with the N-terminal or the C-terminal domain of p50^{cdc37}. Five different constructs were made: 147, 148,149, Ct, and Nt. Two of the constructs, Ct and Nt, were based on previously published data [29] that suggests that hsp90 bound to the C-terminal half of p50^{cdc37}. The 147 construct contained a segment of sequence that overlapped the Ct construct. The various constructs were translated *in vitro* with concomitant radiolabeling and immunoadsorbed with the anti-hsp90 antibody 8D3. The adsorbed p50^{cdc37} polypeptides were detected by autoradiography (Figure 8). Aliquots of unfractionated translations were also analyzed to assess translation efficiency. As previously reported, the Ct gene product strongly bound to hsp90 while the Nt gene product was not detected in anti-hsp90 immunoadsorptions [29]. The 147 polypeptide that overlapped Ct with-in amino acids to164-173 also bound hsp90 but not to the extent of Ct. From this data it seems that the region of p50^{cdc37} from the amino acids 164-173 participates in hsp90 binding. However the 148 and Nt constructs did not translate well enough to assess their potential association with hsp90.

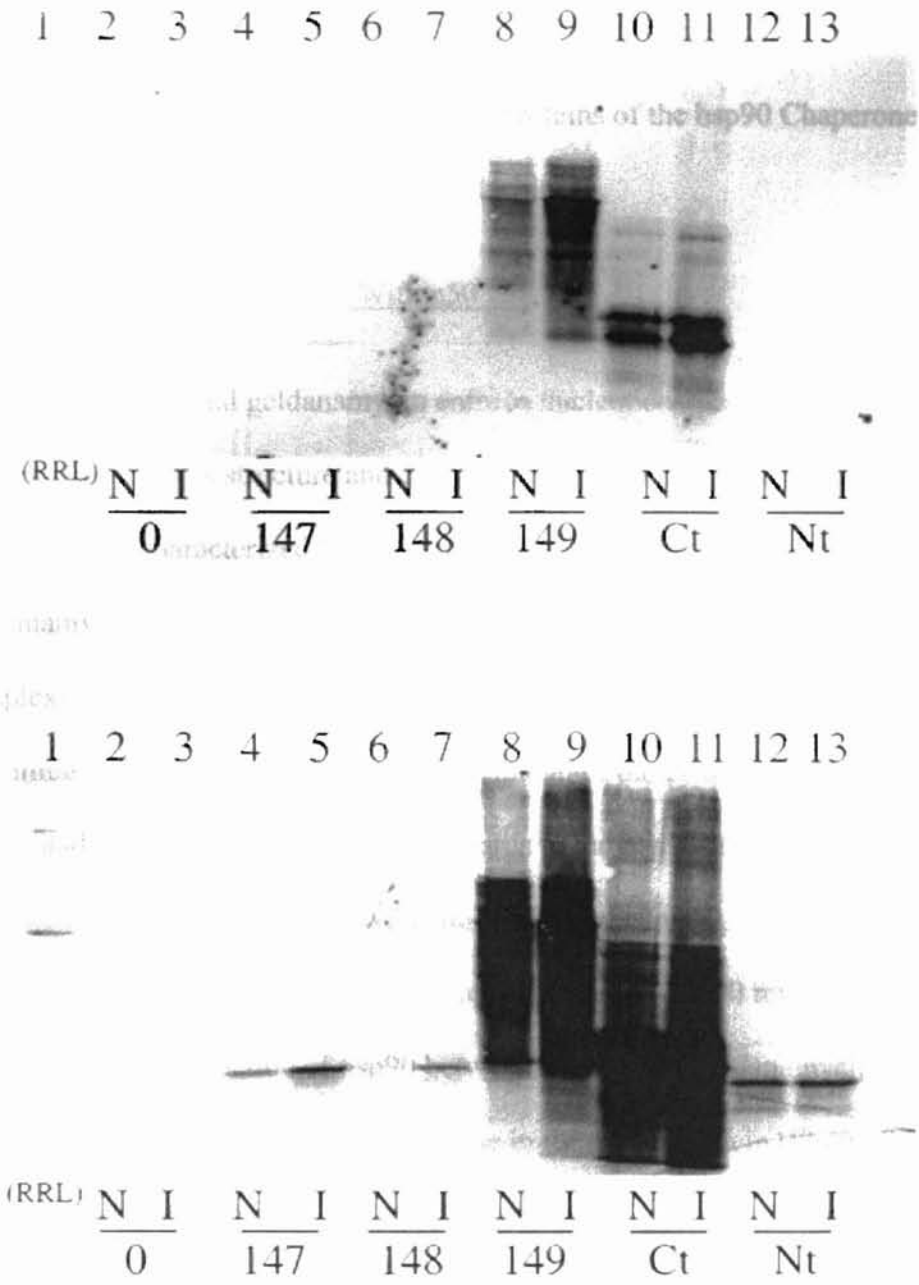


Figure 8. Co-immunoadsorption of hsp90 and Truncated Forms of p50^{cdc37}

Constructs of truncated p50^{cdc37}: 147, 148, 149, Ct and Nt, were *in vitro* translated with concomitant ^{35}S radiolabeling. The resulting translations were immunoadsorbed using the anti-hsp90 monoclonal antibody 8D3. The immunoadsorptions were washed twice with P0T, once with P20T and twice more with P0T. The bound polypeptides were released from 8D3 by the addition of P1000 buffer then mixed with an equal volume of 4x SDS-PAGE SB. The samples were run on 12% SDS-PAGE gels followed by autoradiography.

Interactions Between $p50^{cdc37}$ and the Proteins of the hsp90 Chaperone Complex Machinery

Chaperone Complexes Formed With $p50^{cdc37}$

Molybdate and geldanamycin enforce nucleotide-specific hsp90 conformations, thus altering hsp90's structure and composition. Thus, the proteins of the hsp90 machinery are characterized by which of these drugs enhances its interaction with hsp90. Geldanamycin treatment increases the representation of p60 and hsp70 in hsp90 complexes. In contrast, molybdate increases the recovery of p23 and immunophilins. To determine if the addition of these drugs would alter the association between $p50^{cdc37}$ and hsp90, and help classify $p50^{cdc37}$ with regards to the hsp90-machinery, molybdate and geldanamycin treated PS mixes were immunoadsorbed using anti-$p50^{cdc37}$ antibodies. Western blotting of the immunoprecipitations with anti-hsp90 revealed only slight differences in the detection of hsp90 between the control, geldanamycin and molybdate lanes. However there was a slight increase in the geldanamycin (Figure 9, lane 5) over the control (Figure 9, lane 3) and molybdate (Figure 9, lane 7). Nonetheless, these weak effects did not allow us to conclude that $p50^{cdc37}$ recognizes a specific conformation of hsp90.

To characterize $p50^{cdc37}$'s interactions with hsp90 cohorts, p23 was immunoadsorbed and assayed for associated proteins. When anti-p23 immunoadsorptions (Figure 10) were western blotted for $p50^{cdc37}$, $p50^{cdc37}$ was detected with p23 heterocomplexes in an immune specific manner. To determine if all of the

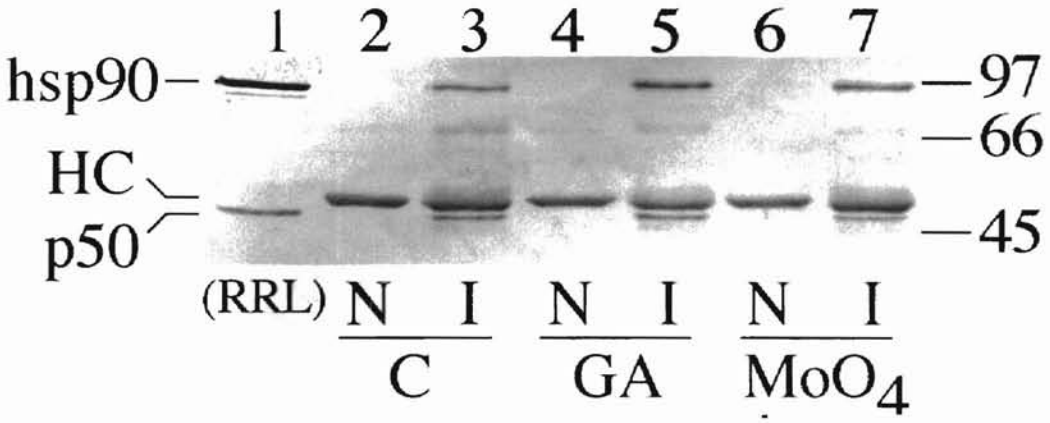


Figure 9. Effects of geldanamycin and molybdate on the physical association between p50^{cdc37} and hsp90.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with anti-p50^{cdc37} (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed four times with P50T (lanes 2-5). Alternatively, molybdate treated lanes were washed with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-p50^{cdc37} and-hsp90 antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

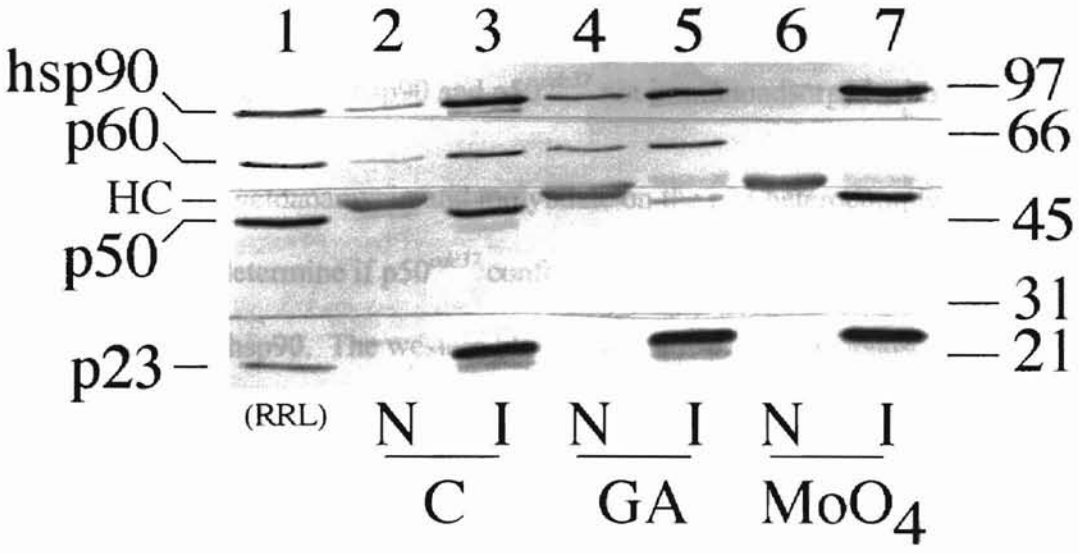


Figure 10. Effects of geldanamycin and molybdate on the physical association between p23 and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with anti-p23 (JJ3) antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Alternatively, molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, p23 *(p23)*, p50^{cdc37} *(p50)* and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

available $p50^{cdc37}$ and hsp90 were bound to p23, an immunodepletion (Figure 3) using the anti-p23 antibodies JJ3 or JJ5 (lanes 5 and 7 respectively) was performed. The results indicated that a significant amount of the hsp90 and $p50^{cdc37}$ are bound to p23 in RRL PS mixes, but there was some hsp90 and $p50^{cdc37}$ not immunoadsorped with p23.

Effects of geldanamycin and molybdate on the p23 heterocomplex were characterized to determine if $p50^{cdc37}$ conforms to the model of early and late cohort interactions with hsp90. The western blots of the anti-p23 immunoadsorption (Figure 11) show a decrease in the detection of hsp90 and $p50^{cdc37}$ in the geldanamycin lane (lane 5) when compared to the control lane (lane 3). However, there is an increase of hsp90 and $p50^{cdc37}$ in the molybdate lane (lane7) when compared to the control. Also seen in the molybdate lane is a lack of detection of p60. These results differ from ones previously reported in that the association between hsp90 and p23 was not completely disrupted when treated with geldanamycin and that the presence of p60 is reduced with molybdate treatment rather than enhanced [37].

To address the differences between the p23:hsp90 complexes that I observed and those of previously published data, experiments to reconstitute the formation of p23:hsp90 complexes after disruption were duplicated [37]. To do this, proteins already present in chaperone complexes in RRL were disassociated by potassium chloride followed by dialysis. After dialysis, RRL PS mixes were or were not supplemented with an ATP regenerating system plus ATP (ARS+ATP). Additionally, molybdate and geldanamycin were added to the RRL PS mixes to assess the conformation –specific association of hsp90 and p23. Treated RRL PS mixes were then adsorbed with JJ3

antibody and coadsorbing hsp90 and p50^{cdc37} were detected by western blotting. The addition of ARS+ATP increased the adsorption of hsp90 relative to the adsorption of hsp90 in ATP deficient protein synthesis mix as previously reported [37]. Additionally, molybdate increased the ATP-dependent association of hsp90 with p23. These results were consistent with those of Jill Johnson, suggesting that hsp90 was quantitatively locked in its alternative configuration by drug treatment under these conditions.

The same salt-stripped lysate was used to reexamine the hypothesis that the reassociation of p50^{cdc37} with hsp90 was sensitive to ATP, geldanamycin and molybdate. Immunoprecipitations of the salt-stripped lysates with anti-p50^{cdc37} followed by the subsequent western blotting for hsp90 and p50^{cdc37} showed no significant differences between the lysate that was supplemented with ATP+ARS and the water supplemented lysates (Figure 12). One of the repeats of this experiment (not shown) did not contain equivalent concentrations of proteins between "ATP+ARS supplemented" and "no ARS+ATP added" because the untreated lysates were not supplemented with an equivalent volume of water, as a consequence less p50^{cdc37} and hsp90 were immunoadsorbed. This is also the case for the molybdate treated lysates compared to the control and GA treated lysates. When this is taken into account the differences between the supplemented and unsupplemented lysates are negligible. Thus, only minor differences in hsp90:p50^{cdc37} association occur as a consequence of ATP, geldanamycin and molybdate. These results indicate that hsp90 and p50^{cdc37} do not require ATP to interact and that p50^{cdc37} does not recognize drug enforced hsp90 conformation.

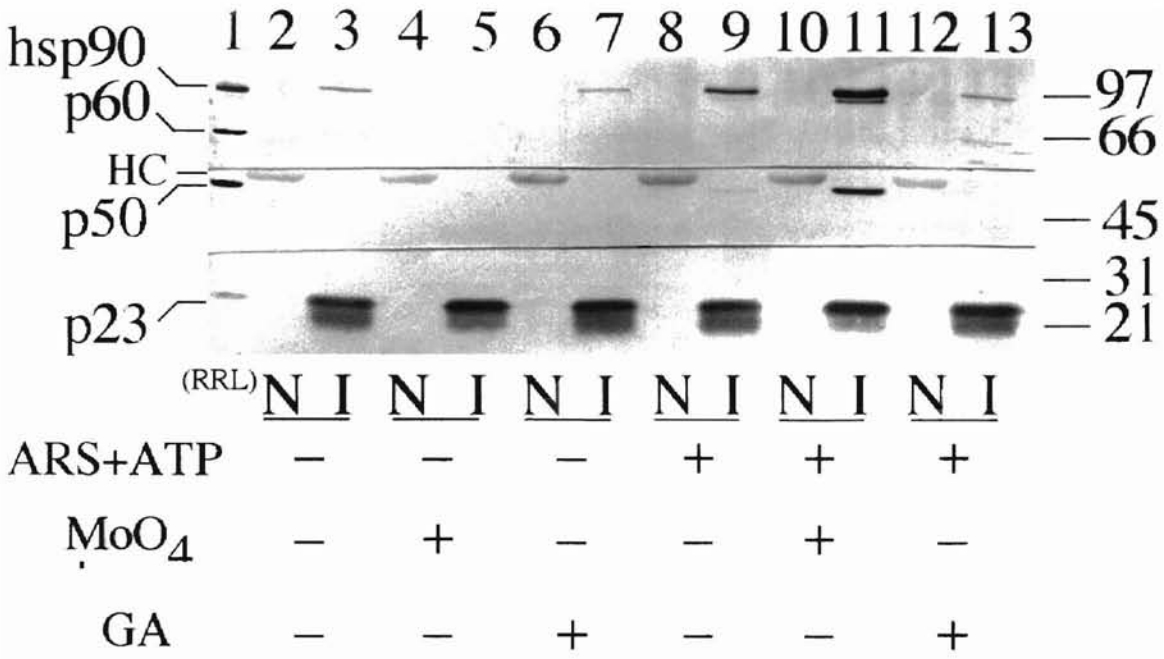


Figure 11. Reassociation of p23 and the hsp90 Machinery in Salt-Stripped Lysate

Salt-stripped rabbit reticulocyte lysate was treated with either water or an ATP regenerating system plus ATP (ARS+ATP) then incubated 5 min at 37° C. Molybdate (MoO_4) or geldanamycin (GA) was added as indicated and the reactions were further incubated at 37° C for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, 6, 8, 10, & 12) or with anti-p23 (JJ3) antibody (I; lanes 3, 5, 7, 9, 11, &13). After binding, the immunoadsorptions were washed five times with P20T (lanes 1, 2, 5, 6, 7, 8, 11, & 12). Molybdate treated lanes were washed five times with P20MT (lanes 3, 4, 9, & 10). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, $p50^{cdc37}$ *(p50)*, p23 *(p23)*, the heavy chain (HC), and the light chain (LC) of the adsorbing antibody are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

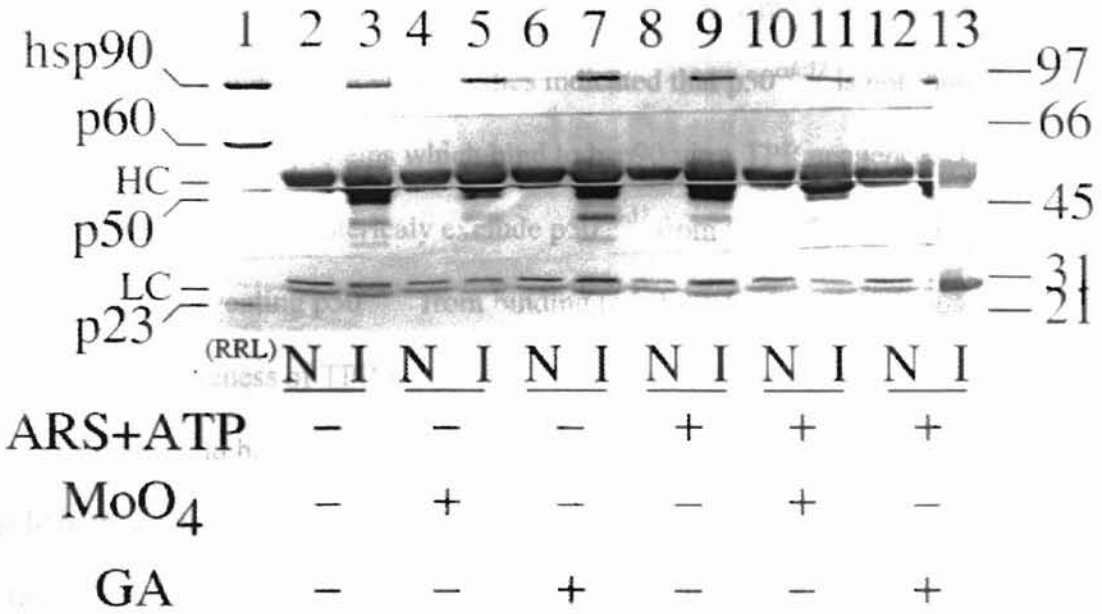


Figure 12. Reassociation of p50^{cdc37} and the hsp90 Machinery in Salt-Stripped Lysate

Salt-stripped rabbit reticulocyte lysate was treated with either water or an ATP regenerating system plus ATP (ARS+ATP) then incubated 5 min at 37° C. Molybdate (MoO_4) or geldanamycin (GA) was added as indicated and the reactions were further incubated at 37° C for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, 6, 8, 10, & 12) or with anti- p50^{cdc37} antibody (I; lanes 3, 5, 7, 9, 11, &13). After binding, the immunoadsorptions were washed five times with P20T lanes 1, 2, 5, 6, 7, 8, 11, & 12). Molybdate treated lanes were washed five times with P20MT (lanes 3, 4, 9, & 10). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, p50^{cdc37} (*p50*), p23 *(p23)*, the heavy chain (HC), and the light chain (LC) of the adsorbing antibody are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

Current models of hsp90 chaperone machinery postulate exclusive cohort compositions bound to hsp90. According to the predominant model, steroid hormone receptor complexes do not include p50^{cdc37} and the kinase/hsp90/p50^{cdc37} complex does not include TPR proteins. Previous studies indicated that p50^{cdc37} is not found in the same hsp90 complex as proteins which bind to hsp90 via a TPR sequence. The bound TPR protein is believed to stericaly exclude p50^{cdc37} from the p50^{cdc37}-binding site on hsp90 thereby preventing p50^{cdc37} from binding to hsp90. One model proposes that the mutually exclusiveness of TPR versus p50^{cdc37} determines the functional difference between the steroid hormone receptors and the kinase/hsp90/p50^{cdc37} complexes. To test the hypothesis that p50^{cdc37} and TPR proteins are mutually exclusive, various hsp90 cohorts were immunoadsorbed and assessed for coadsorption of p50^{cdc37}. Additionally. I collected preliminary data on the effects of geldanamycin and molybdate on these interactions.

Previous studies indicated that purified p60 did not bind to hsp90 that was bound to p50^{cdc37} and that p50^{cdc37} could not bind to hsp90 if TPR proteins were present. To test if p50^{cdc37} is co-adsorbed with p60, anti-p60 (F5) immunoadsorptions were analyzed by Western blotting with anti-p50^{cdc37} antibodies. As expected hsp90 is co-adsorbed with p60 (Figure 13). Surprisingly, p50^{cdc37} was detected in the anti-p60 immunoadsorptions. Previous studies showed that bacterially expressed human p60 competed for p50^{cdc37} binding to hsp90 [8]. Additionally, when densetometry data is compared between the treated and the control lanes, the adjusted volume for hsp90 in the geldanamycin treated lane (lane 5) is similar to that of the control (lane 3), 3.00 compared to 2.96 respectively.

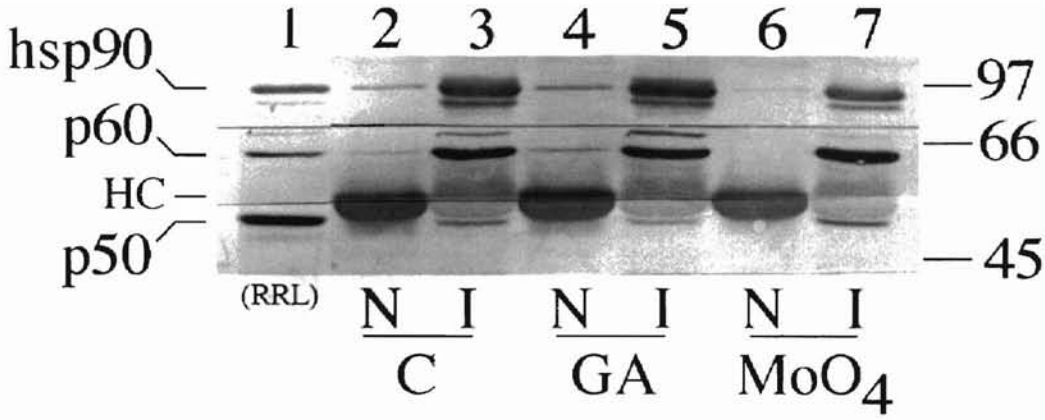


Figure 13. Effects of geldanamycin and molybdate on the physical association between p60 and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with F5, an anti-p60 antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

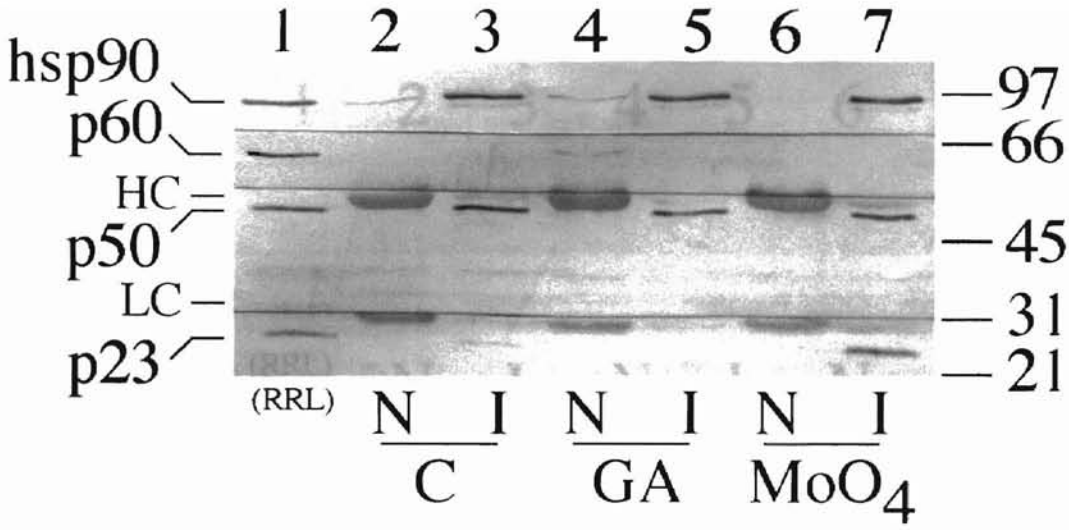


Figure 14. Effects of geldanamycin and molybdate on the physical association between FKBP52 and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with EC1 anti-FKBP52 antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, p23 *(p23)*, p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

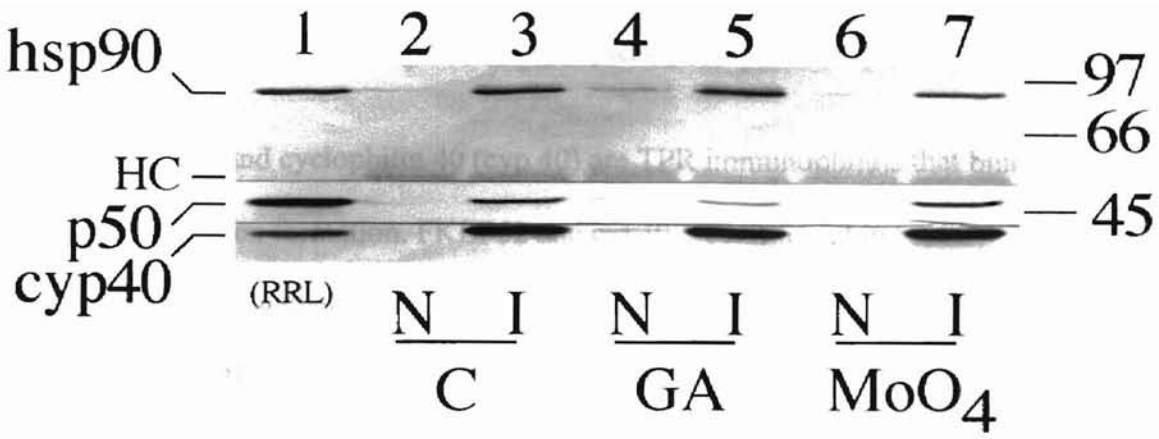


Figure 15. Effects of geldanamycin and molybdate on the physical association between cyp40 and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with anti-cyp40 antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p50^{cdc37} (*p50*), cyp40 *(cyp40)*, and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

However the adjusted volume for p50^{cdc37} in the geldanamycin lane seems to be slightly less then the control lane, 0.298 and 0.611 respectively. On the other hand the molybdate lane (lane 7) has a lower detection of hsp90, 2.79 compared to 2.96, and a higher detection of p50^{cdc37}, 1.00 compared to 0.611, when compared to the control lane (lane 3).

FKBP52 and cyclophilin 40 (cyp 40) are TPR immunophilins that bind to hsp90 during late complex formation. FKBP52 and cyp40 are not found in hsp90 heterocomplexes together. To determine if p50^{cdc37} is found in the same heterocomplex as cyp 40 or FKBP52, immunoadsorptions were performed with either anti-FKBP52 or anti- cyp40 antibodies. Western blotting of anti-FKBP52 immunoadsorptions (Figure 14) with anti-p50^{cdc37} indicate that p50^{cdc37} is found in hsp90 complexes with FKBP52 in an immune-specific manner. The western blots show that less hsp90 and p50^{cdc37} is detected in the geldanamycin lane (lane 5) than there was in the control lane (lane 3). Comparatively the only difference between the molybdate lane (lane 7) and the control lane is a slight increase of p23 detection. When anti-FKBP52 immunoadsorptions were Western blotted with anti-cyp40 antibodies, there was no indication that cyp 40 was present in these complexes consistent with previous reports [8]. These results indicate that the FKBP52:hsp90 complexes do contain p50^{cdc37}, unlike previous studies, but not cyp40 consistent with previous reports [8].

To determine if p50^{cdc37} is also found in hsp90 complexes with cyp40, anti-cyp40 immunoadsorptions were western blotted with anti-p50^{cdc37} and anti-hsp90 antibodies (Figure 15). Again p50^{cdc37} and hsp90 are found associated with a TPR protein, namely

cyp 40, in an immune-specific manner. The geldanamycin lane (lane 5) contained a relatively equivalent amount of detectable hsp90 as the control lane (lane 3); however there is less $p50^{cdc37}$ in the geldanamycin lane compared to the control. Conversely there is less detectable hsp90 in the molybdate lane (lane 7) compared to the control (lane 3). The detectable $p50^{cdc37}$ in the molybdate lane seems to increase over the $p50^{cdc37}$ in the control lane. These results were not consistent with the previously reported hypothesis that $p50^{cdc37}$ is not found in hsp90 heterocomplexes with TPR proteins [8].

To determine if $p50^{cdc37}$ binds to the human DnaJ homologue hsp40, hsp40 was immunoadsorbed with anti-hsp40 antibody and the immunoprecipitations were western blotted for $p50^{cdc37}$, p60, and hsp90. Western blots (Figure 16) showed no detectable hsp90, p60, or $p50^{cdc37}$ in any of the experimental conditions. The band above the heavy chain in the molybdate lane (lane 7) does not appear to correspond to any of the proteins blotted for when compared to the RRL standard lane (lane 1). This is consistent with previously reported data that hsp40 is not found in heterocomplex intermediates or in native heterocomplexes [10].

To determine if $p50^{cdc37}$ binds to the hsp90 cohort hsp70, immunoadsorptions with anti-hsp70 were western blotted for $p50^{cdc37}$ and hsp90. Anti-hsp70 immunoadsorptions did show detectable hsp90 and $p50^{cdc37}$as shown in Figure 17. There appears to be an increase in the detection of hsp90 along with a decrease in the detection of $p50^{cdc37}$ in the geldanamycin lane (lane 5) when compared to the control lane (lane 3). Conversely there seems to be a decrease in the detection of hsp90 and an increase of $p50^{cdc37}$ in the molybdate lane (lane7) when compared to the control. From these results it can be concluded that $p50^{cdc37}$ is found in hsp90 heterocomplexes with hsp70.

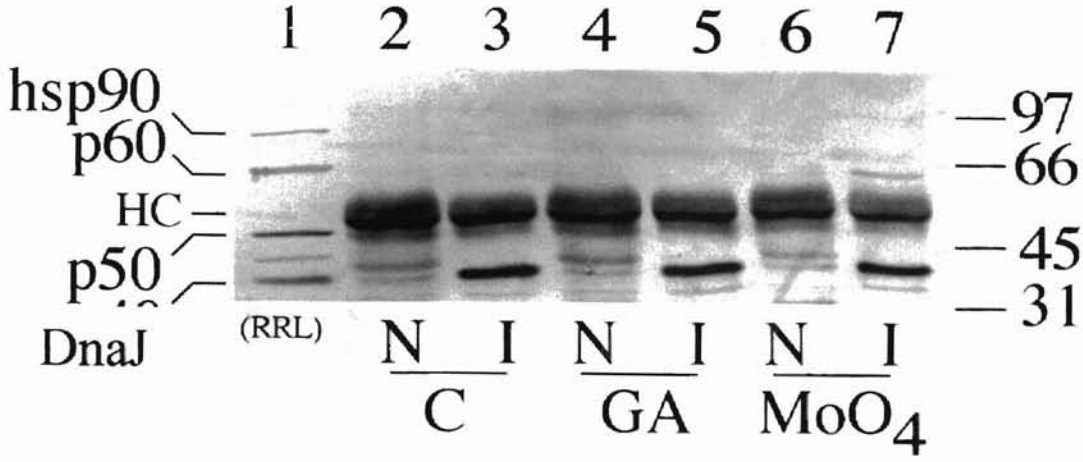


Figure 16. Effects of geldanamycin and molybdate on the physical association between dnaJ (hsp40) and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (MoO_4; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with anti-hsp40 antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), p60 *(p60)*, p50^{cdc37} (*p50*), hsp40 *(hsp40)* and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

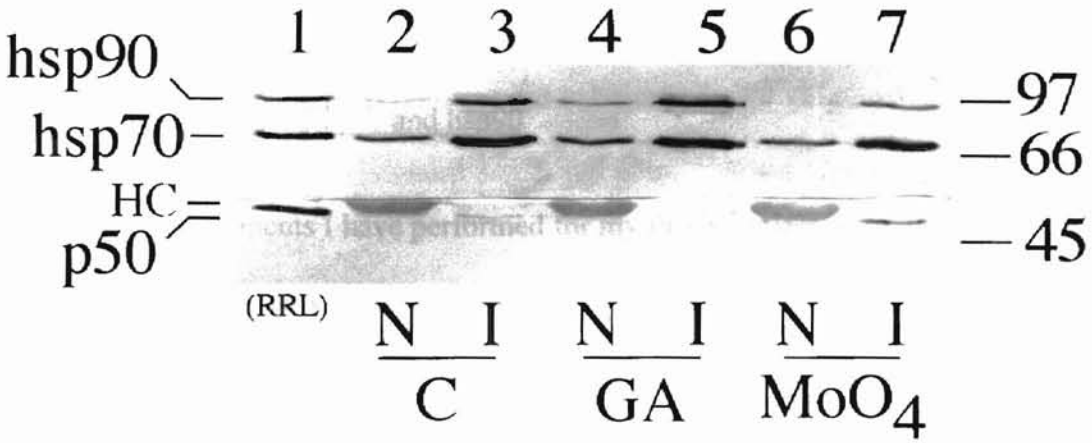


Figure 17. Effects of geldanamycin and molybdate on the physical association between hsp70 and p50^{cdc37}.

Rabbit reticulocyte lysate reaction mixtures containing an ATP-regeneration system were incubated at 37° C. At 5 min geldanamycin (G; lanes 4&5), molybdate (M; lanes 6&7), or water (C; lanes 2&3) was added and the reactions were further incubated at 37° for 10 min. Reactions were then immunoadsorbed with either nonimmune antibody (N; lanes 2, 4, and 6) or with BB70, an anti-hsp70 antibody (I; lanes 3, 5, and 7). After binding, the immunoadsorptions were washed two times with PT, once with P20T and two more times with PT (lanes 2-5). Molybdate treated lanes were washed five times with P20MT (lanes 6&7). Bound materials were eluted by boiling in SDS-PAGE sample buffer and analyzed by western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (*hsp90*), hsp70 *(hsp70)*, p50^{cdc37} (*p50*) and the heavy chain of the adsorbing antibody (HC) are indicated. Migrations of molecular weight markers (kDa) are indicated along the right side of the panel. Lane 1 (RRL) contains an aliquot of RRL protein synthesis reactions loaded as a standard.

CHAPTER IV

DISCUSSION

Interactions Between $p50^{cdc37}$ and hsp90

The experiments I have performed for my thesis project were designed to characterize the kinase-specific component of the hsp90 chaperone machinery, $p50^{cdc37}$. For many years the hsp90 chaperone machinery has been studied using the steroid receptors as substrates. It has also been shown that several kinases form complexes with the hsp90 and that formation of this complex is essential for kinase maturation. It has been proposed that the steroid receptor and the kinases are complexed with distinct hsp90 chaperone machinery, with the main determinant between the two being the appearance of $p50^{cdc37}$ in the kinase complexes but not in the steroid receptor complexes. The hsp90/ $p50^{cdc37}$ /kinase complex is stable in the presence of high salt [8]. However, the interaction between $p50^{cdc37}$ and hsp90 is disrupted by the addition of high salt concentrations (Figure 1). Thus, in the absence of a kinase substrate, it is speculated that $p50^{cdc37}$ interacts with hsp90 via ionic bonding until the kinase substrate is recognized, resulting in a conformational change to allow hydrophobic interactions between hsp90 and $p50^{cdc37}$ and kinase substrate.

Alkaline phosphatase and apyrase did not have significant effects on the hsp90: $p50^{cdc37}$ interaction (Figure 4) suggesting that neither phosphorylation nor dephosphorylation had any effect on the association between hsp90 and $p50^{cdc37}$. To test

if the phosphorylation states are different between p50^{cdc37} bound to hsp90 and unbound p50^{cdc37}, these separate populations were analyzed by vertical slab isoelectric focusing to separate p50^{cdc37} isoforms with small charge differences due to different phosphorylation states [36]. Preliminary results indicate that there is no significant difference in the phosphorylation states between bound and unbound p50^{cdc37} (Figure 5). These data suggest that the interaction between hsp90 and p50^{cdc37} is not dependant on phosphorylation events.

p50^{cdc37} does not recognize a specific nucleotide-regulated hsp90 conformation. The results of apyrase treatment, which degrades ATP to ADP, and salt-stripped lysate treated with geldanamycin, which holds hsp90 in a conformation similar to ADP bound hsp90, show that ATP binding does not have a positive influence on the interaction between hsp90 and p50^{cdc37}. This is further supported by the fact that ATP+ARS was not necessary for the reassociation of the complex after its disruption by salt (Figure 11). The association of hsp90 with of p23 in the complex was assessed as a positive control to confirm that the hsp90 complex was behaving as previously reported when exposed to drug treatments [37] and indicates that the assay was working.

Chaperone Composition of p50^{cdc37} Heterocomplexes

Several observations suggest that p50^{cdc37} is not found in native steroid receptor heterocomplexes that contain one of the tetratricopeptides repeat (TPR) domain proteins. The fact that no TPR proteins have been found in hsp90:Cdk4 complexes, a cyclin-dependent kinase, heterocomplexes that contain p50^{cdc37} lead to the conclusion that p50^{cdc37} was not part of the native hsp90 machinery found in steroid receptor

heterocomplex [38]. Binding studies with TPR proteins or TPR domain fragments were used to determine how their presence affected $p50^{cdc37}$'s binding to hsp90 [8]. It was observed that a molar excess of the full-length proteins did not allow $p50^{cdc37}$ to bind to hsp90, but that the TPR domain fragment did. From these observations it was concluded that the TPR acceptor site of hsp90 must be close to the $p50^{cdc37}$ -binding domain of hsp90. Steric hindrance or physical blockage by the TPR proteins of the $p50^{cdc37}$ -binding site may prevent $p50^{cdc37}$ from binding to hsp90. It was found that bacterially expressed p60, a TPR protein, reduced the binding of both $p50^{cdc37}$ and other TPR proteins to hsp90. Their immunoadsorptions with anti-hsp90 antibodies did show $p50^{cdc37}$ in heterocomplexes with p60, FKBP52, and Cyp40 however they proposed that $p50^{cdc37}$ was directly immunoadsorbed by antibody cross-reactivity instead of being part of the complex. They proposed that p60 was binding to hsp90 complexes that did not contain $p50^{cdc37}$, but they were not able to test this directly by co-immunoadsorption studies because the $p50^{cdc37}$ antibody they used only recognizes the denatured form of $p50^{cdc37}$ [8].

In contrast to the above-mentioned theory, my immunoadsorptions of hsp90 chaperone heterocomplex indicate that $p50^{cdc37}$ binds to hsp90 machinery when TPR proteins are present. Anti-p60 immunoadsorptions showed that p60 was found in a complex that contained both hsp90 and $p50^{cdc37}$ (Figure 13). Similarly, $p50^{cdc37}$ and hsp90 are found in anti-Cyp40 immunoadsorptions in an immune specific manner (Figure 15) and FKBP52 immunoadsorptions contained both hsp90 and $p50^{cdc37}$ (Figure 14). Thus, I concluded that TPR proteins are associated with the hsp90-$p50^{cdc37}$ heterocomplexes. Therefore, the hypothesis that $p50^{cdc37}$'s presence is exclusive versus

TPR proteins is false. However, the appearance of TPR proteins in hsp90/p50^{cdc37} complexes can be reconciled with competition studies if hsp90 functions as a dimer. Hsp90 purifies as a dimer under non-denaturing conditions and it is widely believed to interact with other proteins as a dimer. Thus, although the p50^{cdc37} and TPR binding sites on hsp90 may be adjacent, p50^{cdc37} may be complexed with one hsp90 monomer while a TPR protein is complexed with the other.

p50^{cdc37} is also found in complexes with other hsp90 cohorts including p23 (Figure 10). Immunoadsorption of p23 revealed that p50^{cdc37} was precipitated along with hsp90 in an immune specific manner. To more fully characterize the interactions of p50^{cdc37} with hsp90 and p23, immunodepletions of these proteins were carried out to determine the extent of their interactions with p50^{cdc37}. Immunodepletions of both hsp90 and p23 show that a significant amount of p50^{cdc37} is not found in a stable association with hsp90 and p23. This could indicate that p50^{cdc37} is found free from these proteins. However, this may reflect interaction instability during immunoadsorption due to low protein concentrations or the interactions of p50^{cdc37} with hsp90 and p23 may be unstable and easily dissociated. None the less, there appears to be a significant hsp90-free p50^{cdc37} population.

Geldanamycin and molybdate had complex effects on the amounts of p50^{cdc37} present in the various hsp90 heterocomplexes. The interaction between FKBP52 and p23 is stabilized by molybdate, which is typical for these proteins, however the amount of p50^{cdc37} and hsp90 is constant in all conditions. Immunoadsorptions of p23 treated with molybdate show an increase in the amount of p50^{cdc37}, but with geldanamycin treatment there is a decrease in the amount of p50^{cdc37} detected. The decreases and increases in the

detection of p50^{cdc37} and hsp90 in the p23 and FKBP52 immunoadsorptions seem to be paralleling each other, however the drug effects on the interactions of p60, Cyp40, and hsp70 with p50^{cdc37} and hsp90 are not as straightforward (Figures 13, 15, and 17). There does not seem to be any correlation between amounts of hsp90 and amounts of p50^{cdc37} regarding increases and decreases in detection following drug treatments. When geldanamycin is added to the reaction mixtures before adsorption there is an observable decrease of p50^{cdc37}, but the hsp90 does not seem to be greatly affected. Molybdate had the opposite affect in that hsp90 detection is greatly reduced and the p50^{cdc37} band is similar to the control lane.

Hsp40, the human homologue of DnaJ, is found in hsp90:steroid hormone receptor complexes. Immunoadsorptions of lysates using anti-hsp40 antibodies did not detect any hsp90 or p50^{cdc37} binding. This might reflect the function of hsp40 or hsp40 is isoform specific. Hsp40 is thought to present substrates to hsp70 for binding, therefore only transient interactions with hsp70, and therefore the hsp90 chaperone complexes are expected.

In summary, my data allow me to draw three significant conclusions. The first is that p50^{cdc37} and hsp90 are weakly associated in the absence of a kinase. Additionally, the interaction between p50^{cdc37} and hsp90 is not dependent on phosphorylation events nor does ATP binding to hsp90 effect the interaction. I have also shown that p50^{cdc37} is found in hsp90 complexes along with other hsp90 cohorts. However, more study will be necessary to determine how p50^{cdc37} functions in the hsp90 chaperone machinery.

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Thesis: CHARACTERIZATION OF p50^{cdc37}, THE KINASE-SPECIFIC COMPONENT OF THE HSP90 CHAPERONE MACHINERY

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