HSP90 INTERACTOME

By

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HSP90 INTERACTOME

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ABBREVIATIONS

AA	amino acid
Aha1	activator of the Hsp90 ATPase
ACN	acetonitrile
ADP	adenosine triphosphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BLAST	basic local alignment search tool
Cdc	cell division cycle
Cyp40	40-Kda cyclosporine A-binding protein
DMSO	dimethyl sulfoxide
DTT	dithiothretol
DUF	Domain of Unknown Function
EDTA	ethylenediaminetetraacetic acid
FKBP	FK506 binding protein
Flag-tag	Octa-amino acid peptide-DYKDDDDK
GA	geldanamycin
GHKL	gyrase, Hsp90, histidine kinase, MutL
Hop	Hsp70-Hsp90 organizing protein
Hsp	Heat shock protein
IgG	immunoglubulin G
HspBP1	Hsp70 binding protein1
Lck	lymphoid cell kinase
MALDI-TOF	matrix-assisted laser desorption-ionization time-of-flight mass spectrometer
MS	mass spectrometer
NudC	nuclear distribution protein C
NBT	nitroblue tetrazolium
PP5	Protein Phosphatase 5
PVDF	polyvinylidene difluoride
PCR	polymerase chain reaction
PIPES	piperazine-1,4-bis(2 ethanesulfonic acid)
PMSF	phenylmethanesulfonyl fluoride
PPIase	peptidyl-prolyl isomerases
RRL	rabbit reticulocyte lysate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHR	steroid hormone receptor
Sgt1	small glutamine-rich tetratricopeptide repeat protein 1
Sti1	stress inducible protein-1

TFA	trifluoroacetic acid
TnT	coupled transcription and translation
TPR	tetratricopeptide repeat
TRiC	T complex protein-1 [TCP-1] ring complex

CHAPTER I

Literature Review

Proteins are biological molecules that fold into complex conformations depending on the physical and chemical properties of their amino acid chains (*2*). By folding into a specific three-dimensional shape or a "native" state, a protein is able to perform its biological function (*2-4*). Generally, protein folding is a spontaneous process, but proteins will unfold under denaturing conditions (*2*). Mis-folded proteins form insoluble protein aggregates found in a number of human diseases, such as cancer and neurodegenerative diseases including Alzheimer's and Parkinson's disease (*5*).

In the living cell, some proteins require helper proteins to support their correct and efficient folding. The helper proteins are called stress proteins or heat shock proteins (Hsps) (*6, 7*). They appear when a cell undergoes various types of environmental stresses like heat shock and other cellular stresses. But heat shock proteins are also present in cells under normal physiological conditions.

Heat shock proteins are molecular chaperones for protein molecules. They are ubiquitous cellular proteins and one of the life-guards of the living cells. Differencing from folding catalysts like protein disulfide isomerases etc, molecular chaperones increase the yield of folding reactions by amplifying the number of productive folding pathways, but do not increase the rate of folding reactions (*8-10*). Molecular chaperones perform basic and essential cell functions, such as facilitating newly synthesized

polypeptides to form properly into their biologically native structure, assisting denatured protein to refold, and preventing protein aggregation (*8-10*). Based primarily on their molecular weights and functional mechanisms, there are several different kinds of chaperones (*2*). My research is focused on Hsp90 and its co-chaperones.

Heat Shock Protein 90 (Hsp90)

Hsp90, a highly conserved and ubiquitous chaperone found in eubacteria and all branches of eukarya, is involved in the folding of a set of cell regulatory proteins and in the re-folding of stress-denatured polypeptides (*11*). It is the most abundantly expressed protein in unstressed cell (*12*). Unlike Hsp70, Hsp90 seems to serve as a molecular chaperone for signal transduction proteins, such as steroid hormone receptors (SHRs) and cell-cycle kinases. These proteins are denoted as Hsp90 client proteins. Because many of its client proteins are involved in cell signaling, proliferation and survival, Hsp90 has an essential function in signal transduction pathways (*2, 12*).

Hsp90 plays an important role in cancer because its clients involve in the regulation of the cell cycle, cell growth, cell survival, apoptosis, and oncogenesis (*13*). Previous studies show that level of Hsp90 is higher in various malignant cell lines and cancer cells compared to normal cells (*14*). Many Hsp90 client proteins are known to participate in oncogenic pathways and processes (*15*). Therefore Hsp90 is a potential target of anti-cancer therapy.

In eukaryotic cytoplasm, two distinct genes encode constitutive and inducible isoforms of Hsp90 (Hsp90-α and Hsp90–β). They are closely related and the consequences of gene duplication about 500 million years ago (*1, 2*). They are both

induced by stress and no differences in their activities have been identified (*16*). Homologues of Hsp90 are also found in endoplamic reticulum and mitochondia, named Grp94 (94 kDa glucose-regulated protein) (*17*) and Trap1/Hsp75 (type1 tumor necrosis factor receptor-associated protein) (*18, 19*), respectively. In prokaryotic cells, there is homologue termed HtpG (high temperature protein G) (*20*).

Structure and Function of Hsp90

Biochemical and crystallographic studies have been shown that Hsp90 consists of three major domains (*21-23*): a highly conserved amino-terminal ATPase domain, a middle domain and a carboxyl-terminal dimerization domain (Figure 1)

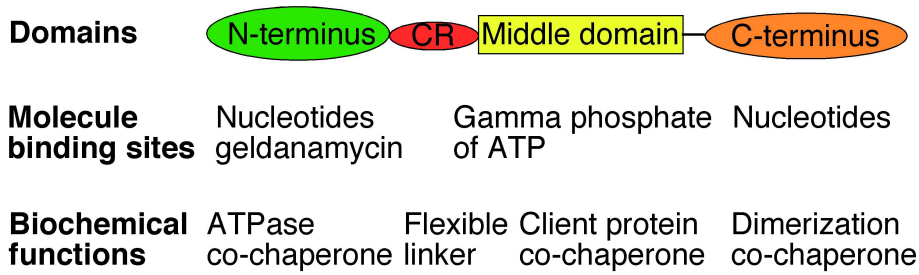


Figure 1: Model for Hsp90 structure and function. 'CR' refers to a red charged region which serves as a linker between N-terminal domain and middle domain. Green N-terminal domain, yellow middle domain and orange C-terminal domain (*14*).

Three-dimensional crystallization structures suggest that the amino-acid terminal domain contains a highly twisted β-sheet exposed on one face, and a set of α-helices covering the opposite face (*2*). At the center of the helical side, a deep pocket forms a binding site for ATP/ADP (*23*). The binding of ATP at the N-terminal site alters the

conformational state of Hsp90 and affects its interactions with client proteins and co-chaperones. This ATP-binding pocket serves as the binding site for the Hsp90 inhibitors geldanamycin and radicicol (*24*). Geldanamycin competes with ATP/ADP in the nucleotide binding pocket, resulting in disruption of the Hsp90 function and degradation of Hsp90 client proteins by the ubiquitin-dependent proteasome pathway (*25, 26*). Like other gyrase, Hsp90, histidine kinase, MutL (GHKL) family members, Hsp90 also requires ATP binding and hydrolysis of ATP to regulate the conformational change (*27*) (Figure 1 N-terminus).

In eukaryotic Hsp90, a highly charged and protease-sensitive segment follows the amino-terminal nucleotide binding domain and connects the N- terminal and the middle domain of Hsp90 (Figure 1: CR) (*28*). Even though this charged region is lacking in prokaryotic HtpG, human Trap1 and Grp94 (*2*), studies indicate that this charged linker region might have a function in regulating Hsp90 chaperone function by increasing affinity of Hsp90 binding substrate and regulating ATP binding to the N-terminal domain (*29*).

The middle domain of Hsp90 also has a key role in the binding of many client proteins to Hsp90 (*2*). Recent studies observe that AHA1, an Hsp90 co-chaperone, enhances Hsp90's ATPase activity by binding to Hsp90 middle domain (*30*). This indicates that the middle domain is also important for modulating ATPase cycle (Figure 1: middle domain).

A second nucleotide binding site appears to be present near the Hsp90 carboxyl-terminal domain, but this is less well characterized (*31-33*). The C-terminus comprises an Hsp90 dimerization site and provides binding site for Hsp90 co-chaperones containing

TPR domains (*34-36*). At the very carboxyl-terminus, all eukaryotic cytosolic Hsp90 have penta-peptide sequence MEEVD. This peptide sequence is the core of interaction between Hsp90 and partner proteins with TPR domain such as immunophilins, Hop/Sti1 and PP5 (*37, 38*). Interestingly, the C-terminal residues of Hsp70 (GPTIEEVD) interact with TPR domains in a manner similar to that of Hsp90 (*37*). Binding of drug to the C-terminal site blocks nucleotide binding to N-terminal site (*31, 33*). This suggests that these two nucleotide-binding sites may interact in coordinated relationship to regulating the Hsp90 conformational change.

The Hsp90 ATPase Activity

ATP binding and hydrolysis are important for Hsp90 function *in vivo*. This conclusion is drawn by the observation that if mutantions inhibit the N-terminal dimerization of Hsp90 that is required for efficient hydrolysis of ATP, these mutants do not support the Hsp90 function (*11, 39*).

The two state models suggest that ATP binding and hydrolysis results in Hsp90 conformation changes. The nucleotide-free state, corresponding to the "open" state of Hsp90 can capture client proteins, with the two N-terminal domains in the dimer being separate. In the ATP-bound state, binding of ATP increases the association of the N-terminal domains putting Hsp90 in the “close” form to clamp the client protein tightly. After ATP hydrolysis, Hsp90 will reverse the conformation change from the “close” to the “open” form and lead to release of the client protein (Figure 2). Therefore, opening and closing of the Hsp90 molecular clamp are coupled to ATPase cycle (*40*).

The antibiotic geldanamycin (GA) is a fungal ansamycin exhibiting anti-tumor activity (*41*). It binds to a conserved ATP-binding pocket in the amino-terminal domain of Hsp90 (*23*). GA binds with higher affinity than the natural nucleotides and prevents the nucleotide-mediated Hsp90 switching between its ADP- and ATP-bound conformation (*14*). Thus, Hsp90 cannot bind tightly to its client proteins. If Hsp90 inhibitor GA binds to Hsp90 *in vivo*, it will induce the proteasomal degradation of Hsp90 client proteins. In contrast to geldamycin, molybdate mimics ATP/ ADP·P state of Hsp90 (Figure 2) and stabilizes the interaction between Hsp90 and client proteins by locking Hsp90 in its high-affinity client-binding conformation (*42*). Therefore, now geldanamycin and molybdate are used as good and rapid tools for screening the Hsp90 client proteins.

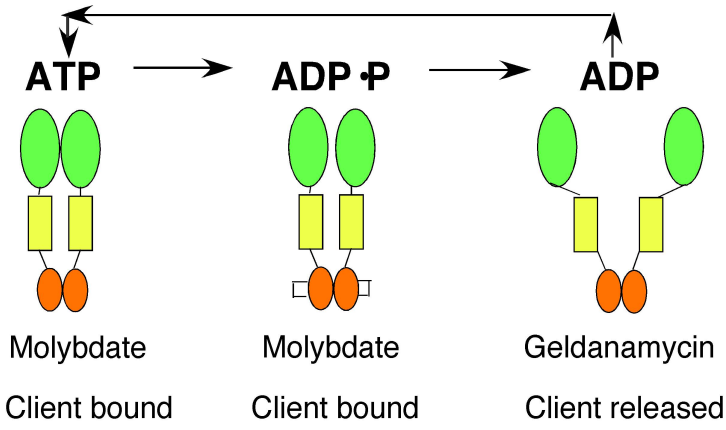


Figure 2: The ATPase-coupled N-terminal dimerization of Hsp90, drug and client binding state.

Hsp90 Co-chaperones

Hsp90 collaborates with its co-chaperones to promote many client proteins folding, functional maturation and stability (*2*). Below, several major Hsp90 co-chaperones will be introduced.

Cdc37 (p50)

Numerous studies have shown that Cdc37 associates with kinase complexes, and then stabilizes and activates many protein kinases by cooperating with Hsp90. Therefore, it is also called "kinase-specific" co-chaperone (*43, 44*). The Cdc37 gene was first identified in yeast as temperature-sensitive cell-division cycle mutation (*44, 45*).

Cdc37 consists of three domains: an N-terminal domain; a middle domain; and a C-terminal domain (*43*). Client proteins (kinases) bind to the N-terminal Cdc37. Hsp90 binds to middle domain of Cdc37. The function of C-terminal domain of Cdc37 is unknown (*46*).

Crystallization of human Cdc37 indicates that Cdc37 blocks the lid of nucleotide binding pocket of Hsp90 in an open position, and inhibits the ATPase activity (*46*). This inhibition results in a certain conformation for client loading. Unlike client protein, co-chaperone Cdc37 binding to Hsp90 does not respond to the effect of geldanamycin and molybdate (*47*).

Cdc37 may also have Hsp90-independent molecular chaperon's activities. *In vitro*, Cdc37 can stabilize inherently unstable kinase (CKII) by keeping unfolded substrates in a refolding form for other chaperones, and behaves like "holding chaperone" (*48*). In addition, Chang's works show that not all of the endogenous Cdc37 is associated with Hsp90 in yeast (*49*). Also it is reported in Hartson *et al.*'s works (*47*).

Co-chaperones Containing Tetratricopeptide Repeat (TPR) Domains

A number of co-chaperones that share a conserved tetratricopeptide repeat (TPR) domain have a common binding site on Hsp90. These co-chaperones are immunophilins (FKBP51 and FKBP52, Cyclophilin 40), Hsp organization protein (HOP), and protein phosphatase 5 (PP5) (*50, 51*). TPR domains contain multiple copies of a degenerate 34 amino acid residues that are arranged in tandem repeat (*52*). Several studies show that the primary binding site for TPR domains is the C terminus of Hsp90 with a conserved C-terminal motif MEEVD (*12*). The work of Gehring *et al*. suggests that several receptor heterocompexes have a heteromeric structure of 1 receptor : 2 Hsp90 : 1 immunophilin (*53-55*).

HOP (Hsp70-Hsp90 organizing protein, p60)

The function of HOP is to facilitate the transfer by binding to both Hsp90 and the substrate-bound Hsp70, forming a physical link between them (*56*). HOP which was identified as a 60 kDa protein (p60), has a yeast homolog which is called Sti1 (*57*). HOP consists of three different TPR domains. TPR1 binds Hsp70, while TPR2a and TPR2b bind to Hsp90 (*58*).

HOP inhibits intrinsic ATPase activity of Hsp90 *in vitro*. The study of Hsp70-Hop-Hsp90 interaction suggests that Hop is not only a physical linker and seems to enhance the activities of these two chaperones, but also blocks ATPase activity by inhibiting the binding of p23 to Hsp90 (*59*). This is due to its inhibition of ATP binding to the N-terminal nucleotide binding pocket of Hsp90 (*59*). Consistent with the above mechanism, HOP binds to the ADP-bound but not ATP-bound form of Hsp90 (*36*). Thus, HOP blocks Hsp90's change to the p23-binding conformation. HOP has also been found

to enhance the ATPase activity of Hsp70 (*60*). This suggests that HOP functions to modulate the ATP cycle of proteins when bringing them together. The interaction between HOP and Hsp90 is enhanced by geldanamycin and is prevented by molybdate (*47*).

Protein Phosphatase 5 (PP5)

PP5 is member of the serine/threonine (Ser/Thr) phosphoprotein phosphatase family (*61*). PP5 has three TPR motifs at its N-terminus and a peptidyl-prolyl isomerase (PPIase) homology domain (*62*). The three TPR domains serve both targeting and regulatory functions.

PP5 associates with steroid hormone receptor (SHR). It binds to Hsp90 via its TPR domain (*63*) and it is a major component of SHR and Hsp90 heterocomplexes (*64*). PP5's phosphatase activity may be important for cytoplasmic-nuclear tracking.

Immunophilins

Like PP5, the three high molecular weight immunophilins with TPR domains-FKBP51, FKBP52, CyP40 bind to steroid receptors and Hsp90 complexes. According to binding to different immunosuppressant drugs (FK506 or cyclosporine), they generally can be characterized into two groups: the FKBPs binding with FK506 and rapamycin, and the cyclophilins (CyPs) binding with cyclosporine A (*51*). Immunophilins also have a role in helping SHR maturation and trans-activation for the chaperone activity of Hsp90 (*65*). Like Cdc37, immunophilins binding to Hsp90 also do not respond to the effect of geldanamycin and molybdate (*47*).

p23

p23 stabilizes protein-Hsp90 hetero-complex ATP-dependent interaction. It is the smallest protein in the Hsp90 machine (Mr = 23 kDa). p23 is a ubiquitous and highly conserved protein from yeast to humans. It plays a role in facilitating the adenosine triphosphate–driven cycle of Hsp90 binding to client proteins. It enters at a late stage of the Hsp90 cycle (*66*) and stabilizes steroid receptor together with Hsp90. And it also enhances the client complexes in maturation state. Biochemical studies indicate that ATP and p23 cause rearrangements in Hsp90 heterocomplexes which are important for the progression of the chaperone cycle (*67, 68*). The interaction between p23 and Hsp90 is dependent on ATP binding (*69*), i.e., p23 binds to the ATP-bound form of Hsp90 when Hsp90 achieves the ATP-bound conformation (*67*). Geldanamycin, a specific inhibitor of Hsp90 that competes with ATP for binding, can disrupt the Hsp90-p23 complex (*23, 24*). But the molybdate intensifies the association of Hsp90 and p23 (*47*).

Activator of Hsp90 ATPase 1 (*AHA1)*

Aha1 is a newly described Hsp90 co-chaperone. It binds to the middle domains of Hsp90 (*30*). Thus, Aha1 does not compete for binding C-terminal domains of Hsp90 (*70*). Instead it only competes with the early cofactors Hop and p50, but can bind to Hsp90 in the presence of cyclophilins, suggesting that Aha1 acts as a late cofactor of Hsp90 (*71*). Panaretou's studies demonstrate that *in vivo* Aha1 is not only specific for kinase activation, but also regulates the maturation of hormone receptors, and has a function in the activation of Hsp90-dependent client proteins (*67*).

Hsp90-Substrate Interaction

The function of Hsp90 is to facilitate proper folding of substrate proteins. However, Hsp90 cannot support the function of biologic substrates such as steroid receptors on its own and requires the assistance of co-chaperones (*3*). Studies suggest that the jobs of some co-chaperones are specifically linked to substrate protein processing. To date, Hsp90 substrates include steroid hormone receptors, transcription factors, tyrosine and serine/threonine kinases and other signal transduction proteins(*2*). Generally, co-chaperone immunophilins are typically characterized as participants of steroid-receptor-Hsp90 complex assembly, and Cdc37 is most commonly found in kinase-Hsp90 complex.

The exact mechanism how Hsp90 recognizes and assembles all the client proteins properly folding is still unclear. However, extensive studies on steroid hormone receptors indicate that co-chaperones interact with Hsp90 in a sequence-ordered fashion and form a cyclic chaperone pathway. According to this model, Hsp70 binds the client (steroid hormone receptor) first on its own. Subsequently, Hsp70 and client are delivered to Hsp90 and the scaffold protein Hop. They fold into an "intermediate chaperone complex". After ATP hydrolysis, chaperone Hsp70 and Hop components dissociate. At this time, p23 and immunophilins enter the complex and form a final complex. Then the client protein is released from this cycle (Figure 3) (*2*). After release, the protein may become independent, or it may re-enter a reiterative cycle.

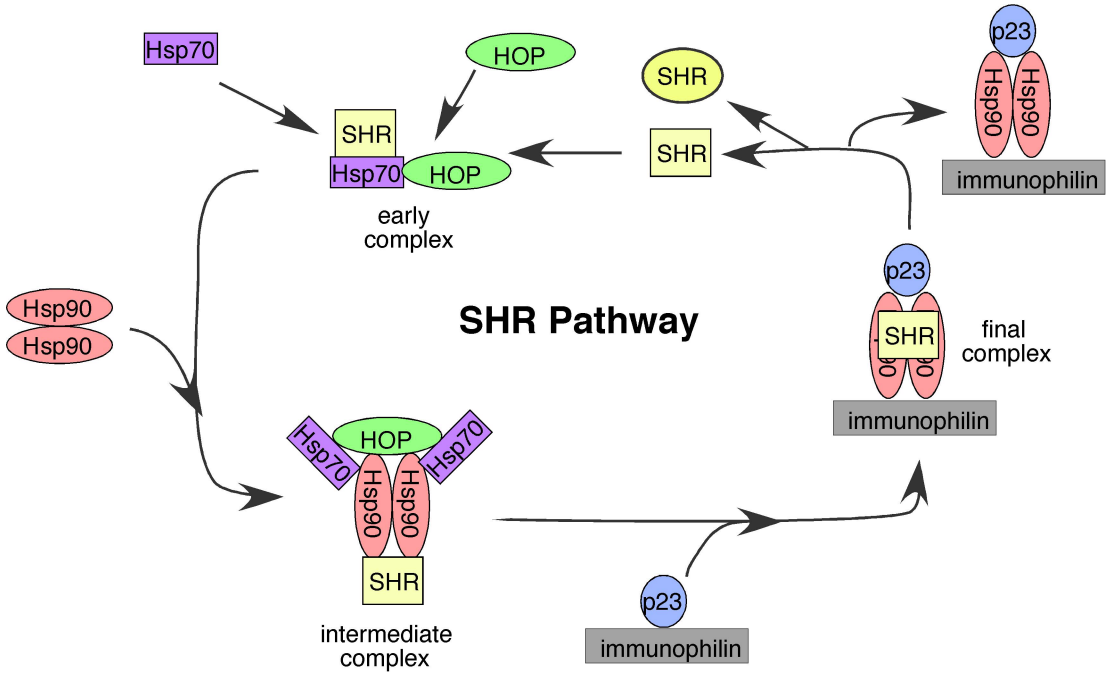


Figure 3: Basic chaperone cycle for activation of steroid hormone receptors (*2*).

CHAPTER II

Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2), A Novel Hsp90-Partner Protein

2.1 Introduction

Hsp90 assembles into large multi-protein complex including many co-chaperones, partners and clients (*14*). Hsp70, one of the major chaperones, helps fold newly synthesized polypeptide chains and participates in assembly of many multiproteins (*2*). Chaperone Hsp40 stimulates Hsp70 ATPase activity, while activator of the Hsp90 ATPase (AHA1) stimulates Hsp90 ATPase activity (*71*). The function of Hsp70-Hsp90 organizing proteins (HOP) is to mediate association between Hsp90 and Hsp70 (*58*). HOP acts like adapters. CDC37/p50 interacts with kinase (*72*), immunophillin modulates association with hormone receptors (*38*). p23 stabilizes interaction of Hsp90 and clients (*3*).

Geldanamycin, a nature product antibiotic, inhibits the chaperone activity of Hsp90 by binding N-terminal ATP-binding pocket of Hsp90. Geldanamycin binding to Hsp90 has higher affinity than the natural nucleotide, so it stabilizes drug binding and prevents Hsp90 conformational switching during its normal ADP- to ATP-bound cycle. Geldanamycin inhibits client binding to target, and causes client degradation *in vivo*. Due

to its physiological and biochemical effects, geldanamycin is used as a good tool for the study of Hsp90 complexes (*23, 24*).

A novel protein strongly associates with anti-Hsp90 antibody immunoadsorption from the lysates in Jurkat T-cell (Hartson and Te, in preparation; Figure 4). Based on the result of mass spectrometry and database search at the NCBI or SwissProt, Hartson and Te identified this protein as Human Brain Protein 16 (HBP16, Gene ID: 51236, NCBI: AAH09915.1). Until now, nothing has been published on Human Brain Protein 16. Because the name "Human Brain Protein 16" only simply describes cDNA library source from RNA isolation, and is not informative, as well as other reasons shown in section 2.3, we propose to change the name "Human Brain Protein 16" to Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2). The data from Hartson and Te's Hsp90 co-immunoadsorption suggest that ARCBP2 physically interacts with Hsp90.

In this report, we confirm identify of ARCBP2 and also present evidence that ARCBP2 acts as a novel Hsp90 co-chaperone partner.

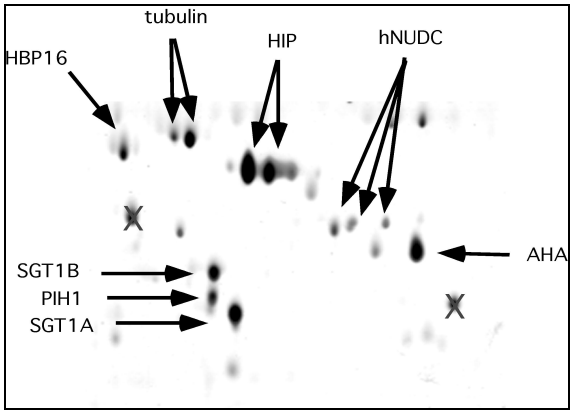


Figure 4: Hsp90-associated proteins displayed on 2-D Gels. Jurkat T-cells were lysed. Lysates were immunoadsorbed with mouse monoclonal anti-Hsp90 antibody (8D3). The proteins were displayed on 2-D Gel. "X's" are proteins bound to non-immune control antibody resins. Small glutamine-rich

tetratricopeptide repeat protein 1B (Sgt1B) and nuclear distribution protein C (NudC) were recently described as Hsp90 partners by our lab. PIH1 was identified as an Hsp90 partner in Yeast. AHA and HIP are known co-chaperones. *Figure courtesy of Hartson and Te.*

2.2 Materials and Methods

Reagents. Cell culture medium and supplements were from Invitrogen Corporation. Fetal bovine serum (FBS) was from Atlanta Biologicals. His-select nickel affinity gel was obtained from Sigma. NHS-activated sepharose TM 4 fast flow was purchased from Amersham Biosciences. 8D3 monoclonal IgM antibody to Hsp90 was provided by Dr. Gary H. Perdew. Mouse monoclonal IgM antibody against Hsp90 (3G3) was purchased from Affinity BioReagents (catalog MA3-011). The anti-Flag M2 antibody was purchased from Stratagene (catalog 200472). Control antibodies, mouse IgM (TEPC 183) and mouse IgG1 (MOPC21) were obtained from Sigma. His-tag antibody was from Qiagen. Alkaline phosphatase-conjugated affinity rabbit anti-mouse IgG antibodies were from Jackson ImmunoResearch.

Immunoresin wash buffer contains: 10 mM PIPES pH 7.0, 150 mM NaCl and 0.1% Tween 20 (P150T); 10 mM PIPES pH 7.0, 500 mM NaCl and 0.1% Tween 20 (P500T); 10 mM PIPES pH 7.0, 1000 mM NaCl (P1000); 10 mM PIPES pH 7.0, 20 mM NaCl (P20): and 10 mM PIPES pH 7.0, 100 mM NaCl and 0.1% Tween 20 (P100T).

Plasmid Construction. A full length ARCBP2 cDNA clone was obtained from Open Biosystems (catalog number MHS 1011-60159; Genbank#: BC003035.1). The ARCBP2 ORF was amplified and engineered by standard PCR cloning techniques. For expression in *Escherichia coli*, the PCR products were digested with *Bsmb1* and *Xho1*

and ligated complementary ends of *Nco1*- and *Xho1*- digested bacterial expression vector pET-30a (+). The PCR primer established the reading frame to match the sequence of the His-tag. Open reading frame integrity was checked by DNA sequencing. Thus, the resulting plasmid, pETARCBP2, contains the full-length ARCBP2 with an epitope tagged open reading frame in an orientation that transcription with T7 RNA polymerase yields a sense RNA. The resulting protein had the predicated sequence MHHHHHHSSGLYPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAM_1, with the underlined residues indicating the tag and final methionine being the authentic ARCBP2 start codon (made by Te, Lab accession#: H104).

For *in vitro* translation, expression constructs encoding epitope-tagged (His-) ARCBP2 were created by PCR. The PCR products cut with *Bsmb1* and *Xho1* and ligated into complementary ends of *Nco1*- and *Sal1*- digested pSP64THlck vector. So the construct, pSPARCBP2, has a full length ARCBP2 open reading frame in an orientation such that SP6 RNA polymerase yields a sense RNA. This resulting protein had the predicated sequence MRGSHHHHHHGSM_1, with the underlined residues indicate the tag, and the final methionine being the authentic ARCBP2 start codon (made by Te, Lab accession#: H101).

For *in vivo* transfection, the PCR templates using Flag-ARCBP2 in pSP64T vector (Lab accession#: H102), were trapped into TOPO vector, digested by *HindIII (*mimic *Bsmb1)* and *ApaI*, and then ligated into mammalian expression vector (pcDNA). The plasmid ARCBP2, has the full-length ARCBP2 with an epitope-tagged open reading frame in an orientation such that T7 RNA polymerase with CMV promoter. The resulting protein had the sequence MDYKDDDDKTSM_1, with the underlined residues indicating

the Flag tag, and final Met being the authentic ARCBP2 start codon (made by letong, Lab accession#: H132).

Protein Purification of His_6-ARCBP2 from E. coli. Procedure for ARCBP2 protein purification is that described by Qiagen. pET- His_6-ARCBP2 was expressed in *E. coli* DH5-alpha strain. Cells were harvested, lysed and sonicated in NaH_2PO_4 buffer (PH 8.0) containing 300 mM NaCl and 10 mM imidazole. Cell lysate was mixed with small pinch DNase at room temperature for 30 min. Supernatant was mixed with 4 ml (pact volume) His-select nickel affinity resin (Qiagen) for 3 hours at 4 °C, then the mixture was poured into a column and washed with 400 ml wash buffer containing 50 mM NaH_2PO_4, 15 mM imidazole and 0.3 M NaCl. His_6-ARCBP2 protein was eluted from this resin with this same buffer containing 250 mM imidazole. After elution, peak fractions were pooled and dialyzed against HEPES buffer containing 20mM HEPES pH 8.0, 5mM $MgCl_2$, 500mM KCl and 0.1% Tween. Then aliquots were stored in -20 °C or liquid nitrogen until further use.

Western Blot. Western blotting of whole T-cell lysates (TCL) was performed by standard techniques. After separation by SDS-polyacrylamide electrophoresis (SDS-PAGE) gel, immune complexes were transferred to a polyvinylidene difluoride (PVDF) membrane and visualized by staining with coommassie blue R-250 and/or exposed to X-ray film. Then the membrane was probed with primary antibody, washed with TBS buffer and probed with appropriate secondary antibody (alkaline phosphatase-conjugated secondary antibody) at room temperature for 2 hours. Finally the membrane was developed with 5-bromo-4-chloro-3-indonly-phosphate (BCIP) and nitroblue tetrazolium

(NBT). The results were digitized using a scanning densitometer, and quantified by "BioRad MultiAnalyst" program.

Generation of [^{35}S]ARCBP2 Protein in RRL. To examine the interaction of newly synthesized ARCBP2 with Hsp90, ARCBP2 was synthesized by coupled transcription-translation *in vitro*. The plasmids of Flag- and no-tag ARCBP2 were used to program 30-50 μl nuclease-treated rabbit reticulocyte lysates in [^{35}S]Met for 30 min at 30 °C. For samples treated with drugs, geldanamycin (GA, final concentration of 0.02 μg/μl) or an equal volume of dimethyl sulfoxide (DMSO) for control were added into above reactions. DNA template was omitted for a negative control reaction.

Co-Immunoadsorptions of Protein Complex. Anti-mouse IgG immunoresin was coupled to anti-Flag antibody. Anti-rabbit IgG immunoresin was coupled to rabbit anti-ARCBP2 antibody for 2 hours at 4 °C. Then the immunoresins were washed once with P150T, once with P500T, once with P1000, and two times with P20. Immunoadsorptions always were performed on ice. Before doing co-immunoadsorption, immunoadsorptions were clarified by centrifugation for 10 min at 10krpm. After that, immunoresins were mixed with 50 μl translation reactions stirred on ice for 2 hours. Or immunoresins with 300 μl K562 cell lysates were rocked at 4 °C for 2 hours. Then immunocomplexes were washed 5 times with P100T. Finally, the samples were boiled in sodium dodecyl sulfate (SDS) sample buffer. As negative control, RRL without DNA templates was also assessed in adsorptions at the same time.

Complexes were separated on 8% SDS-PAGE gel. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane, stained with coommassie blue R-250, analyzed by autoradiography and probed by Western blotting as indicated.

Analysis of the Effect of GA on ARCBP2 Levels in Human Cell Line. Human K562 erythroleukemia cells were grown in six-well plates for 20 hours at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotics to approximately 1×10^7 cells/ml. Cells were treated with either DMSO or 0.1 μM geldanamycin for 24 hours. After cell pellets were washed once with Hanks buffer, they were then boiled in SDS-sample buffer, diluted and separated on 10% SDS-PAGE gels. Membranes were blotted with anti-ARCBP2.

Human Jurkat cells were similarly treated with 0.1 μM geldanamycin or same volume of drug vehicle (DMSO) for 20 hours. Cell pellets were washed with PBS and boiled in SDS sample buffer. The samples were loaded and separated on SDS-PAGE gel. After that the membrane was probed with anti-ARCBP2. The Hsp90-dependent kinase Lck was used for a positive control to show that GA has an effect on Hsp90 and function.

Preparation of Cell Lysates. K562 cells were grown in RPMI 1640 media containing FBS and were transfected with DMRIE-C following the manufacturer's instructions. After 48 hours, cells were harvested. Cell pellets were washed once in Hank's buffer, and then lysed in 0.5% Igepal, 20mM Hepes (pH 7.4), 100mM NaCl, 2mM EGTA, 1 mM DTT, 10% glycerol, 1mM $NaVO_4$, and 2x mammalian protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation at 13,000×g for 10 min at 4 °C.

In-Gel Trypsin Digestion. The SDS-PAGE gel of ARCBP2 complexes was destained in 50% methanol/10% acetic acid for several hours until the gel background was clear. This gel then was soaked in clean water. The acrylamide bands containing the proteins of interest were excised. Gel fragments were washed three times with 50%

acetonitrile (ACN)/25mM ammonium bicarbonate (pH 8.0), mixed by rocking 2 hours each, then dehydrated by soaking in 300 µl 100% ACN. Gel pieces were dried completely in a speed vacuum centrifuge, rehydrated in 30 µl of 12.5ng/µl trypsin prepared in 50 mM ammonium bicarbonate (pH 8.0). After incubation at 37 °C overnight, the digested fragments were extracted from the gel with 30 µl of 0.1% trifluoroacetic Acid (TFA). The samples were extracted twice for 1 hour at room temperature. The extracted supernatant was pooled together and subsequently concentrated in a speed vacuum centrifuge to a final volume of 5-10 µl.

MALDI-TOF Mass Spectrometry Analysis. Saturated α-cyano-4-hydroxycinnamic acid (matrix) was prepared in 50% acetonitrile/0.1% TFA. 0.7 µl aliquot of the concentrated peptide mixture was spotted onto the MALDI plate, which was immediately covered by the same volume of 1/2 matrix working solution (50% saturated matrix: 50% ACN/0.1% TFA). Peptide mass standards were also spotted nearby. After being dried completely, peptide samples were analyzed using matrix-assisted laser desorption-ionization time-of-flight mass spectrometer (MALDI-TOF). Proteins were identified by Mascot search in MSDB20050701 Data Base. Search were carbamidomethyl (C), oxidation (M), propionamide (C), and pyro-glu (N-term Q) modifications; Unrestricted protein mass; +/- 100 ppm peptide mass tolerance; 1 max missed cleavage.

2.3 Results

ARCBP2 From Other Species. Since the function of ARCBP2 is unknown, we tried to predict the possible function of ARCBP2 by searching for other similar proteins. The BLAST search showed that proteins similar to ARCBP2 were present in many

species: mouse (E value: 3e-138), fish (E value: 3e-92), and Arabidopsis (E Value: 3e-15). High E value had highly conserved sequences. These conserved sequences mean that they might have an essential conserved function in these species. Among these conserved sequences, there are no known functions.

The NCBI Conserved Domain database search (Figure 5) identified two conserved domains of unknown Function (DUF) on C-terminal of ARCBP2. Because DUF383 and DUF 384 domains were only shown on ARCBP2 protein, they were not seen in other domain architectures. Therefore protein architecture searches suggested that DUF 383 and DUF 384 were not widely distributed among different proteins family.

Another conserved domain is found in N-terminal of ARCBP2. This domain contains Armadillo/beta-catenin-like repeat (ARM) motif (E value: 7e-05). ARM motif exists in many different proteins and consists of 40 amino acids long tandemly repeated sequences. These tandem repeats form a super-helix of helices to mediate protein-protein interaction. This is one reason that we changed the name of Human Brain Protein 16 to Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2).

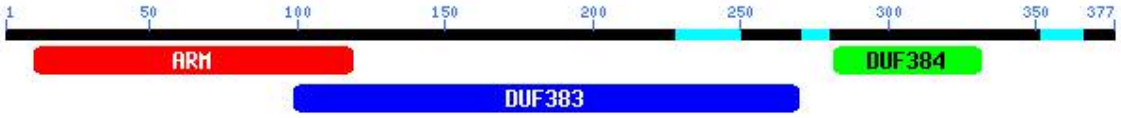


Figure 5: Conserved domain structure of Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2).

BLAST conserved domain searches used to analyze the sequence of ARCBP2.

In order to find conserved motifs, six putative ARCBP2 sequences from mouse, yeast, zebra fish, fly, human and Arabidopsis were used to do alignments (Figure 6). Clustal W alignments illustrate that ARCBP2 had highly conserved sequence on C-

terminus and less conserved sequence on N-terminus. The sequence in human was almost identical to that in mouse. The ARCBP2 sequence was approximately 40% conserved among these different species, especially at C-terminus. This sequence construct suggests that the N-terminal function of ARCBP2 is to bind protein, but the c-terminal function of ARCBP2 is totally unknown.

Identification of Protein Associated with ARCBP2. Since the function of ARCBP2 is wholly novel, we attempted to find and identify associated proteins by protease digestion technique with MALDI-TOF mass spectrometry analysis. As shown in Figure 7, the result indicated that the molecular weight of bands were about 55-60 kDa. Peptide mass fingerprinting identified that these proteins were subunits of the eukaryotic chaperonins containing T-complex protein 1 (CCT, S43062), T-complex protein 1-theta subunit (TCP-1-theta, P50990) and TCP1 ring complex protein (TRiC5, A38983). These results are only preliminary data report from MASCOT search. Because these mass spectrums should be carefully compared to peptide-peptide by protein sequences and database search, these data need to be further confirmed again. Three associated proteins are eukaryotic cytoplasmic “chaperonin” chaperones that facilitate folding of tubulins and actin. ARCBP2’s interaction with chaperone subunits is another reason that we changed HBP16 to Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2).

Formatted Alignments

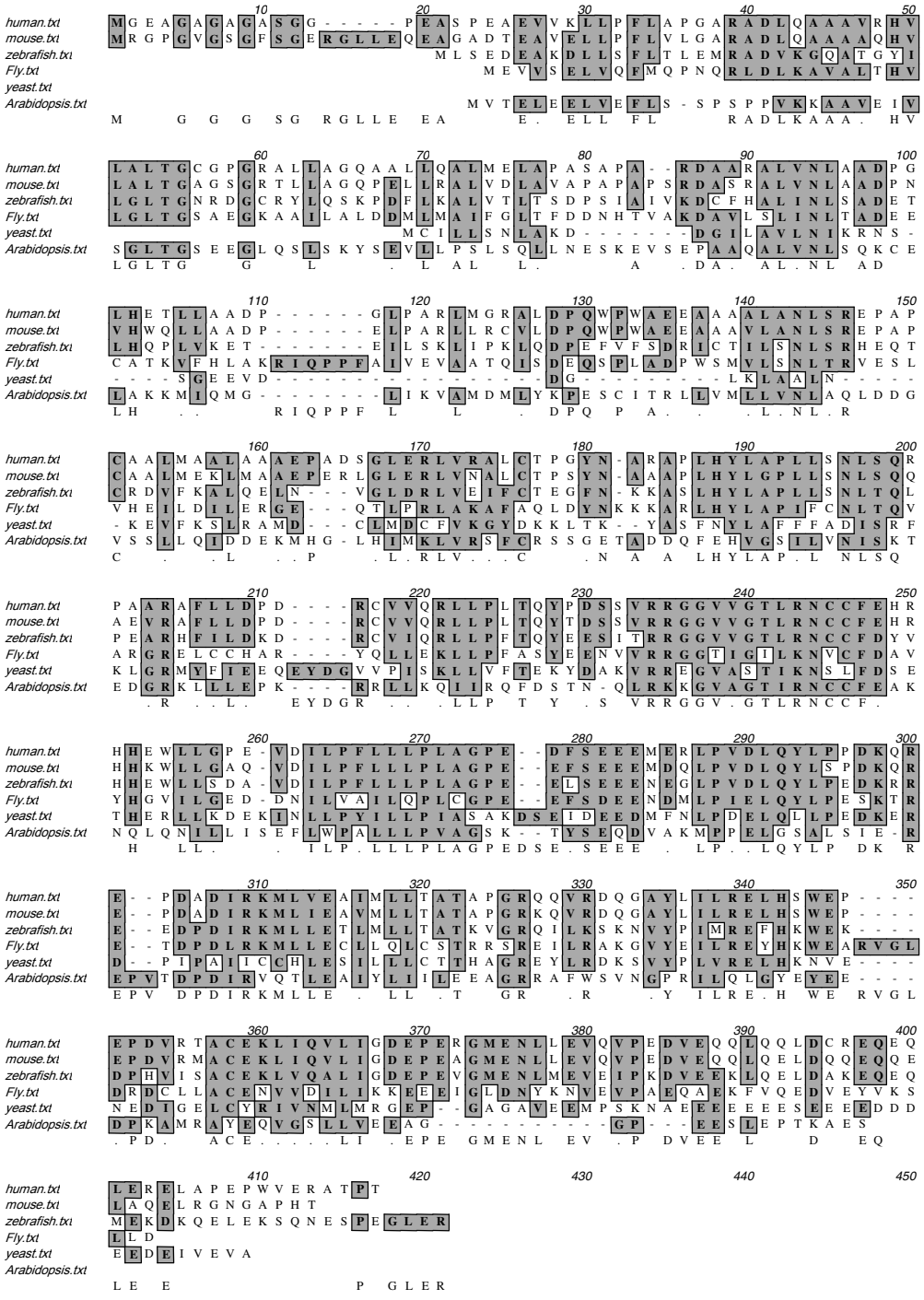


Figure 6: Clustal W Alignment of six different ARCBP2 homologs: human (AAH09915), yeast (AAA77038), mouse (AAH61251), zebra fish (NP001002522), fly (EAL28299) and Arabidopsis (BAC42050).

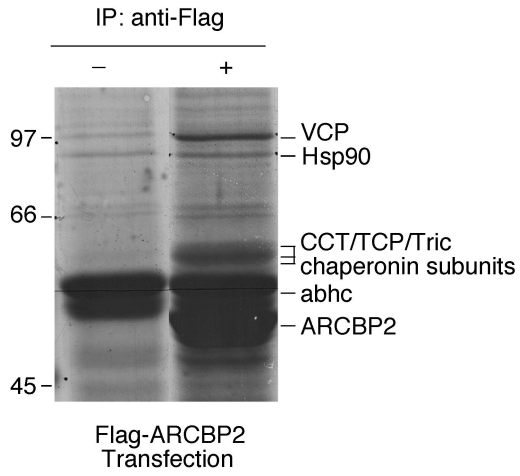


Figure 7: ARCBP2-associated proteins displayed on 1-D Gels. K562 Cells transfected with plasmid encoding Flag-ARCBP2, lysed and immunoadsorbed on SDS-PAGE Gel. These bands were identified by mass spectrometry and NCBI database search.

The Physical Interaction between ARCBP2 and Hsp90. Anti-Hsp90 antibody (8D3) immunoadsorbs a protein identified as ARCBP2 from cell lysates (Hartson and Te, in preparation). This might result from direct recognition of ARCBP2 by the monoclonal anti-Hsp90 antibody, or from the physical association between ARCBP2 and Hsp90. To differentate these possibilities, two kinds of monoclonal anti-Hsp90 antibodies (8D3 and 3G3) that recognize different Hsp90 epitopes, were used to co-immunoadsorb Hsp90.

Hsp90 heterocomplexes were isolated by immunoadsorption with 8D3 antibody and blotted with anti-ARCBP2. The data showed that when endogenous Hsp90 heterocomplexes adsorbed from lysates of Jurket T-cell, associated ARCBP2 protein was detected by Western blot. The co-adsorped ARCBP2 protein was specific relative to

control antibody co-immunoadsorption (lane 1 versus lane 2&3). This result is not only consistent with previous study that ARCBP2 and Hsp90 were in same complexes, but also confirms that identify of Hsp90 protein is ARCBP2.

Again, immunoadsorption with 3G3 antibody showed that ARCBP2 was immunospecifically detectable in the Hsp90 complex. Moreover, the amount of ARCBP2 that adsorbs with Hsp90 is proportional to the amount of Hsp90 recovery and repetition. Because both 8D3 and 3G3 antibodies can adsorb ARCBP2, this result confirms that the association between Hsp90 and ACRBP2 is not epitope cross-reaction or antibody artifact.

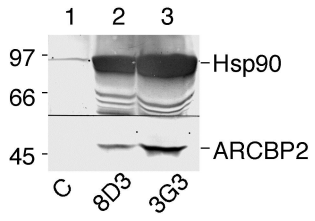


Figure 8: Complex formation between Hsp90 and ARCBP2 in Jurkat cell lysates. The mouse monoclonal anti-Hsp90 antibodies 8D3 and 3G3 (lanes 2&3) or irrelevant non-immune antibody as control (lane 1) were used to immunoprecitate protein heterocomplexes from Jurkat T cell. Washed immunoadsorptions were Western blotted with anti-Hsp90 (top panel) or anti-ARCBP2 antibody (bottom panel).

To confirm that ARCBP2 is present in Hsp90 heterocomplexes, we performed the reciprocal analysis. Anti-ARCBP2 antibody was used to immunoadsorb ARCBP2 and immunopellets were Western blotted for co-immunoadsorption of anti-Hsp90. Results showed that Hsp90 was immuno-specifically associated with ARCBP2 complexes (Figure 9)

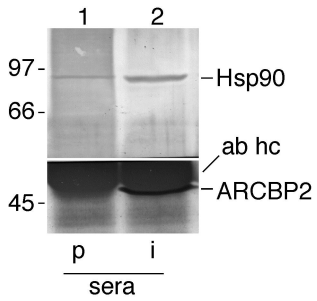
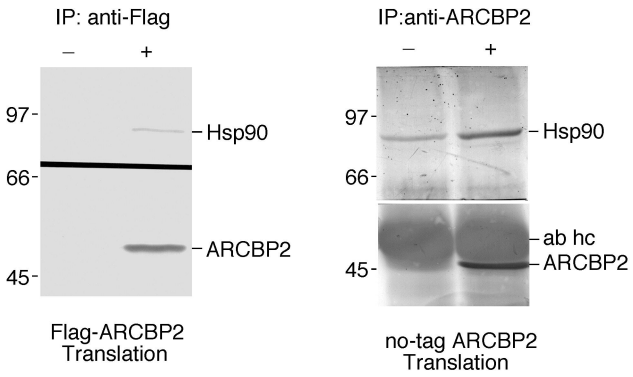


Figure 9: Co-adsorption of Hsp90 with anti-ARCBP2 Antibodies. Rabbit antibodies were used to immunoadsorb lysates of Jurkat cells, and adsorptions were analyzed by Western blotting with anti-Hsp90 (top panel) or anti-ARCBP2 (bottom panel). Hsp90, antibody heavy chains (ab hc), ARCBP2, and migrations of molecular weight markers are indicated. Lane 1, immmunoadsorption with pre-immune antibodies (p sera); lane 2, immunadsorption with antibodies from rabbits immunized with recombinant ARCBP2 (i sera).

To further confirm the interaction between ARCBP2 and Hsp90, ARCBP2 was translated *in vitro*. Co-immunoadsorption with affinity Flag-tagged antibody via using DNA encoding epitope-tagged (Flag-) was performed in rabbit reticulocyte lystate. The result showed that Hsp90 was detected specifically (Figure 10A).

Consistent with above result, antiARCBP2 antibody also co-immunoabsorbed Hsp90 with untagged ARCBP2 in RRL (Figure 10B). Hsp90 is immunospecific compared to control. Since Hsp90 is very sticky protein, there is a non-specific band on the control lane.



A B

Figure 10: Immunoadsorption of ARCBP2 associated with Hsp90. Tagged or untagged ARCBP2 were synthesized in rabbit reticulocyte lysate using transcription-translation, and immunoadsorbed with antibody against the affinity tag (α-Flag, A) or ARCBP2 (B). (+) indicated sample programmed with ARCBP2 plasmid. The control for non-specific binding was native reticulocyte containing no DNA template (-). Immunoadsorptions were Western blotted with anti-Hsp90 and anti-ARCBP2 antibodies, as indicated.

We extended our analysis by immunoadsorbing ARCBP2 and its associated proteins from the human cell line K562. K562 cells were transfected with a construct encoding Flag-tagged ARCBP, ARCBP2 complexes were isolated by anti-flag antibody immunoadsorption, and adsorptions were probed for the presence of Hsp90. The result showed that Hsp90 was specifically recovered with ARCBP2. Compared with negative control, this absorption is specific and valid (Figure 11). This experiment again demonstrated that ARCBP2 physically interacts with Hsp90 in both human and mammalian cell lysates.

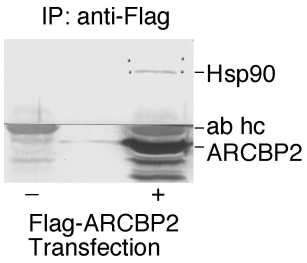


Figure 11: Co-immunoadsorption of Hsp90 and ARCBP2 from transfected K562 cells. K562 cells were transfected with plasmid encoding affinity-tagged ARCBP2 (Flag-ARCBP2). (+) Indicated sample programmed with Flag-ARCBP2 plasmid. (-) indicated a negative control from cells that were no DNA template. Cells were harvested, lysed and immunoadsorbed with antibodies directed against the affinity tag (anti-Flag). Immunopellets were probed with anti-Hsp90 (top panel) and anti-ARCBP2 (bottom panel). The antibody heavy chain is indicated as ab hc.

Characterization of the Effect of Geldanamycin (GA) and Molybdate (MoO_4) on ARCBP2 protein in RRL. To determine whether ARCBP2 is an Hsp90 client or partner, we tested the effect of GA and molybdate on the association of Hsp90 and ARCBP2. Geldanamycin (GA) is a specific anti-cancer Hsp90 inhibitor, which blocks ATP binding in Hsp90's nucleotide-binding pocket. This binding prevents nucleotide-mediated switching of Hsp90 conformation. Thus, Hsp90 cannot bind tightly to its client protein. In contrast to geldanamycin, molybdate stabilizes interaction of Hsp90 with the client proteins and with the p23 co-chaperone by locking Hsp90 in its high-affinity client-binding conformation. Thus, we treated the RRL with GA and molybdate. ARCBP2 complexes were isolated by immunoadsorptions with antibody directed against epitope-tagged ARCBP2. The data showed that the density of Hsp90 band on GA treated lane was weaker than that on DMSO treated lane. However, in the presence of molybdate, the density of Hsp90 band was stronger than control (Figure 12). Therefore GA inhibited the association of ARHBP and Hsp90, but the molybdate enhanced this interaction. This

result indicates that biochemical association of ARCBP2 with Hsp90 complexes behaves like an Hsp90 : client interaction or like a p23 : Hsp90 chaperone interaction.

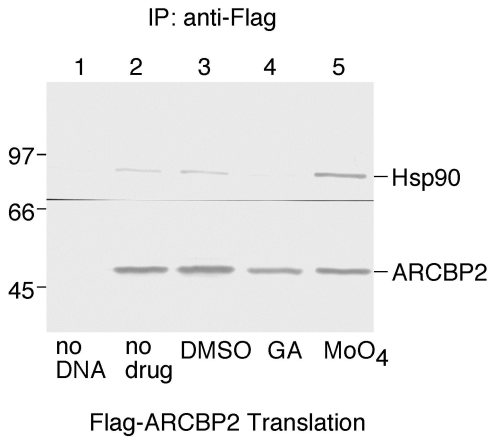


Figure 12: Effect of GA and MoO_4 on ARCBP2 : Hsp90 complexes. Flag-ARCBP2 was synthesized in rabbit reticulocyte lysates in presence of 0.1 µM GA (lane 4) or an equal volume of DMSO (lane 3) for 30 min at 30 °C. Lane 5 was treated with 20 mM sodium molybdate for another 5 min. Reactions were then chilled, immunoadsorbed with anti-Flag antibody. The immunoadsorptions (lane 1, 2, 3 & 4) were washed five times with buffer containing 100 mM NaCl, and the immunoadsorptions (lane 5) were washed five times with buffer containing 80 mM NaCl and 20 mM sodium molybdate. Washed immunopellets were subjected to Western blotting with anti-Hsp90 and anti-ARCBP2 antibodies. The control for non-specific binding was native reticulocyte lysate with no DNA template (Lane 1). Lysate treated with no drug (lane 2) was used to be a negative control.

Characterization of the Impact of GA on ARCBP2 Levels in Living Cells. To determine the interpretation that ARCBP2 is an Hsp90 client or p23-like Hsp90 chaperone, we characterized the impact of geldanamycin on ARCBP2 levels in living cells. GA inhibits the function of Hsp90 and causes known Hsp90 clients to degrade *in vivo*. If ARCBP2 is Hsp90 client, the stability of ARCBP2 in K562 cells was predicted to require its interaction with Hsp90 and its co-chaperones, and thus to be degraded in GA

treated cells. As shown in Figure 13, ARCBP2 levels had no significant differences between DMSO versus GA treatments. This stability was inconsistent with the hypothesis that ARCBP2 behaves as an Hsp90 client.

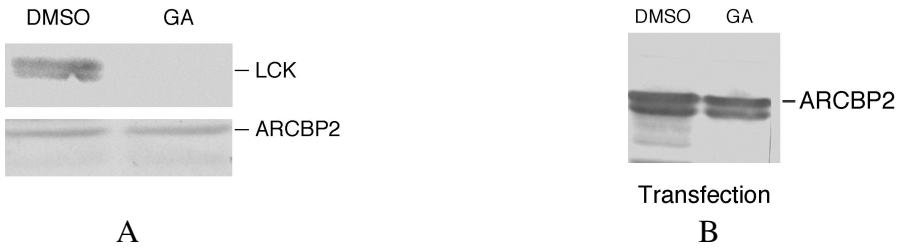


Figure 13: Effects of GA on ARHBP2 levels in living cells. (A) Jurkat cultures were treated with 0.2 μM geldanamycin (GA) or equal volume of drug vehicle (DMSO) for 18 hours. Cells were lysed in SDS sample buffer and immunoblotted with anti-ARCBP2 and anti-lck (positive control). (B) Flag-ARCBP2 plasmids were transfected into K562 cell. The cells were then cultured in the presence of 0.2 μM GA and equal volume of DMSO for 20 hours and directed against ARCBP2 antibody.

2.4 Discussion

The results from characterizations indicate that ARCBP2 is an Hsp90 binding protein. There are five evidences: (1) Two different anti-Hsp90 monoclonal antibodies specifically co-adsorb ARCBP2 from extract of endogenous T-cell. (2) The amount of ARCBP2 that absorbs with Hsp90 is directly proportional to the amount of Hsp90 recovery. (3) Reciprocal co-immunoadsorption with the ARCBP2 antibody co-adsorbs Hsp90 endogenous complexes. (4) Antibody against the Flag affinity tag co-immunoadsorbs Hsp90 from the cell lysates containing Flag-ARCBP2, but not from control lysates lacking. (5) Both antibodies against the Flag affinity tag and directly against ARCBP2 can specifically co-immunoadsorb Hsp90 from rabbit reticulocyte lysates. These data build strong and convincing argument for the association between

ARCBP2 and Hsp90 in mammalian cell lysates. This interaction also indicates there is an association in vivo.

Functionally, our results do not support designating ARCBP2 as an Hsp90 client. Up to date, all classical Hsp90 clients are degraded in response to Hsp90 inhibition by geldanamycin *in vivo*. Moreover, Hsp90 inhibitor geldanamycin prevents association of clients with Hsp90 (*73*), while molybdate strengthens this interaction *in vitro (42)*. Unlike clients, the level of ARCBP2 protein in the living cells (K562 & Jurket cell) did not change significantly in the presence and absence of geldanamycin compared to the positive control Lck kinase, a known Hsp90 client (Figure 13). This result indicates that ARCBP2 is not an Hsp90 client, i.e., Hsp90 is not required for the proper folding for newly synthesized ARCBP2 or for maintaining the stability of ARCBP2 protein. Consistent with it, all the captured proteins displayed in 2 D gel (Figure 4) are Hsp90 partners (Hartson and Te, in prepararion), and there are no Hsp90 clients seen. Furthermore the amount of the ARCBP2's band is similar to the amount of other co-chaperones, such as FKBP52 (Figure 4).

Because ARCBP2 did not undergo geldanamycin-induced degradation *in vivo*, ARCBP2 instead acts like an Hsp90 co-chaperone protein. Generally, Hsp90 co-chaperones can be divided into three groups according to response to the effect of geldanamycin and molybdate, One group is "the early co-chaperone" HOP and Hsp70. The interaction of these two chaperones with Hsp90 is enhanced by geldanamycin and is prevented by molybdate. In contrast, "late co-chaperone" p23 belongs to a second group. Geldanamycin poisons the formation of high-affinity interaction within Hsp90 and p23.

Molybdate intensifies this association. As a third group, CDC37/p50 and immunophilin do not respond to geldanamycin or molybdate (*47*).

Just like p23 co-chaperone, Hsp90 function or the interaction between Hsp90 and ARCBP2 protein is inhibited by geldanamycin, while this association is enhanced by molybdate. However, *in vivo* ARCBP2 protein is not degraded by Hsp90 inhibitor geldanamycin (Figure 14). Thus, the results in this report demonstrate that ARCBP2 physically interacts with Hsp90 via pull-down assays and subsequently ARCBP2 is verified to be a novel Hsp90 partner - p23 like co-chaperone.

ARCBP2 has three major domains: DUF 384, DUF 383 and ARM domains (Figure 5). Even though the function of DUF 383 and DUF 384 is unknown, we found ARCBP2 homolog in yeast HGH1p (function unknown protein). This is consistent with Dr. Houry's work that HGH1p had been identified to be an interacting partner of yeast Hsp90 (*74*). HGH1p is similar to human high mobility group proteins 1 and 2 (HMG1 & HMG2) based on the NCBI database search. Chromatin non-Histone HMG1 and HMG2 are 27 and 25 kDa members of a family of proteins containing multiple HMG boxes. The multiple HMG boxes are conserved domains of 80 amino acids that mediate DNA binding of many proteins. HMG box domains recognize DNA structure, such as four-way junction DNA and non-specific DNA sequences. Both HMG1 and HMG2 contain an N terminal HMG box, a central HMG box, and an acidic carboxyl terminus. The acidic tails of these proteins contain multiple serine residues that match the phosphorylation consensus sites of casein kinase II, and phosphorylation of this domain appears to be important for proper functioning of these proteins. HMG1/2 has been shown to facilitate the binding of various sequence-specific transcription factors to their respective DNA

binding sites. HMG1/2 may serve as architectural factors that recognize and mediate DNA structural changes that accompany various events such as DNA repair, transcription and replication (data not shown). This information suggests that possible function of ARCBP2 is to mediate binding of different protein to Hsp90 clients, which also bind to DNA, such as hormone receptors.

Arm domains consist of 40 amino acid Armadillo (ARM) repeat, which was first identified in the Drosophila segment gene product Armadillo. Armadillo is homolog of mammalian β-catenin. The ARM repeat has a common origin with the HEAT repeat. Both ARM and HEAT repeats have seven highly conserved hydrophobic residues. ARM domain appears to function in binding with target proteins. Animal Arm-repeat protein performs various processes including intracellar signaling and cytoskeletal regulation. In addition, there is another Armadillo repeat chaperone called Human Hsp70 binding protein 1 (HspBP1) (*75*). Shomura's work suggests that the HspBP1 protein with armadillo domain bind to Hsp70 chaperone machinery, as well as it responds to Hsp70 ATP conformational change (*76*). ARCBP2 also has armadillo domain, but it binds to another chaperone Hsp90 and responds to the specific Hsp90 conformation.

The primary structure and the protein association of ARCBP2 suggest that ARCBP2 acts as an adaptor subunit to help protein-protein interaction. Although the function of ARCBP2 is unclear, from the data shown here we can speculate that ARCBP2 behaves as an Hsp90 adapter analogous to HOP. ARCBP2 might mediate association between Hsp90 and the mammalian chaperonin TriC/CCT (Figure 7). Studies of cooperation between different chaperones are critically important in protein folding pathway. TRiC (TCP1-ring complex, also called CCT) is chaperonin in the eukaryotic

cytosol. Different from Hsp70, the function of TRiC is to bind nonnative polypeptide in its central pocket and facilitate their folding in an ATP-dependent manner (*77*). Recent studies of protein interactions in *S. cerevisiae* suggested that TRiC/CCT associates with a large number of proteins containing oligomeric complexes (*77*). The chaperone helps oligomer assembly. Recent studies show that steroid receptor is a hetero oligomeric complex, which needs Hsp90 assistance. ARCBP2 is found to associate with both Hsp90 and chaperonin TRiC/CCT (Figure 7), indicating that ARCBP2 may act like an adaptor and may have a role in supporting the chaperones to maintain the correct folding and stability of client protein.

According to our results, I speculate that ARCBP2 not only is a simple Hsp90 adaptor like HOP, but also it behaves as Hsp90 co-chaperone like p23. ARCBP2 binds to Hsp90 in a certain conformation, which is inhibited by GA and enhanced by molybdate. That means that ARCBP2 probably has a role in the binding of Hsp90-bound client protein. Alternatively, association of Hsp90 and TRiC with ARCBP2 might require a defined order of chaperone interactions. We need to test the sequence order of ARCBP2 binding to Hsp90 and TRiC chaperone system, or what kind of a client binds to ARCBP2, the relationship between Hsp90, client and ARCBP2, and the function of TRiC. These mechanisms need to be done in future.

In summary, our results demonstrate that ARCBP2 physically interacts with Hsp90 and recognizes specific Hsp90 conformations. This novel chaperone partnership may link Hsp90 and TRiC in a pathway of chaperone-assisted protein proper folding.

CHAPTER III

Is 15-Lipoxygenase-1 A Novel Hsp90 Binding Protein?

3.1 Introduction

Lipoxygenase (LOX) is an enzyme that plays a pivotal role in the synthesis of inflammatory mediators known as leukotrienes. It belongs to a family of non-heme iron containing dioxygenases that induce peroxidation of polyunsaturated fatty acids. LOX exhibits regiospecificity during interaction with substrates and on this basis have been designated as arachidonate 5-, 8-, 12-, 15-lipoxygenase. Four different enzymes insert oxygen at carbon 5, 8, 12 or 15 of aracidonate acid.

Of particular interest is 15-lipoxygenase (15-LOX) since it has been implicated in the pathogenesis of atherosclerosis, as well as it is an important enzyme during erythroid cell differentiation. There are two 15-LOX isoenzymes, 15-LOX-1 and 15-LOX-2. The preferred substrate for 15-LOX-1 is linoleic acid and for 15-LOX-2 is arachidonic acid (*78*).

15-LOX-1 participates in the break-down of internal membranes by peroxidizing esterified polyunsaturated fatty acids in membranes, thus modifying the structure and function of lipid-protein complexes(*79*). This is why RRL has a lot of 15-LOX-1. Previous studies suggest that over-expression of 15-LOX-1 had been found in human tumors (*80*). 15-LOX-1, a fat-metabolizing enzyme, correlates with the cancer progression and tumorigenesis (*80, 81*).

15-LOX-1 was recently identified in protein complexes containing Hsp90 (Shao, Hartson & Matts, unpublished). In this report, I characterize 15-LOX-1 interaction with Hsp90.

3.2 Materials and Methods

Reagents. K562 and Jurkat cells were obtained from ATCC. Rabbit polyclonal 15-Lipoxygenase-1 (15-LOX-1) antibody was purchased from Cayman Chemical (catalog No. 160704). Antibody recognizing poly-His epitope tags was from QIAGEN (catalog 34660). Polyclonal mouse antibody to Hsp90 was previously described (*82*). Protein G sepharose resin was from Sigma. Alkaline phosphatase-conjugated anti-mouse IgG antibodies were from Jackson ImmunoResearch.

Immunoresin wash buffer contained: 150 mM NaCl, 0.1% Tween 20 and 10 mM PIPES pH 7.0, (P150T); or 500 mM NaCl, 0.1% Tween 20 and 10 mM PIPES pH 7.0 (P500T); or 1000 mM NaCl and 10 mM PIPES pH 7.0 (P1000); or 20 mM NaCl and 10 mM PIPES pH 7.0 (P20) and 100 mM NaCl, 0.1% Tween 20 and 10 mM PIPES pH 7.0 (P100T).

Plasmids. Full-length 15-lipoxygenase type 1 cDNA clones were obtained from ATCC (catalog # ATCC7262911; Genbank # BC029032). DNA encoding 15-LOX-1 was amplified and engineered by standard PCR cloning techniques. Constructs were cloned via *BspmI* and *EcoRI* into a modified pSP64T plasmid encoding a C-terminal His_6 tag for *in vitro* translation as previously described. Thus, the resulting plasmid, pSP64TLOX (made by Te, Lab accession#: H151), contains the full-length 15-LOX-1 with an epitope-tagged open reading frame in an orientation such that SP6 RNA polymerase yields a sense RNA. This produced DNA coding for recombinant 15-LOX-1 sequence

MRGSHHHHHHGSM$_1$, with the underlined residues indicate the His tag and the final Met indicates the authentic LOX start codon. Forward priming sequence showed that His-LOX-1 was perfect in frame.

For expression in mammalian cell, the full-length 15-LOX1 PCR products were amplified. The product was digested with *HindIII* and *EcoRI* and ligated into expression pcDNA3.0 vector. This generated the construct, pCDNALOX, having the full-length untagged LOX in an orientation that transcription with T7 polymerase from the CMV promoter yields a sense RNA. Integrity of the whole sequence was checked by DNA sequencing (made by letong, Lab accession#: H149).

Cell culture and Transfection. K562 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C to approximately 1×10^7 cells/mL. Plasmid 15-LOX1 was transfected into K562 cells in 6-well plates using DMRIE-C transfection reagent according to manufacturer's instructions. For the controls, DNA was omitted from transfections. After 48 hours cells were harvested, and washed once in Hanks buffer, then lysed as described in Chapter II. The samples were clarified in a micro-centrifuge at 14,000×g for 10 minutes.

Co-Immunoadsorptions of Chaperone with His-tagged LOX-1. The construct of His-LOX1 was used to program nuclease-treated rabbit reticulocyte lysate (TnT) containing [^{35}S]Met via coupled transcription/translation for 30 min at 30 °C. After synthesis the samples were treated with 20 mM molybdate for another 5 min. DNA template was omitted for a negative control reaction.

Before immunoadsorption, all immunoprecipitation samples were clarified by centrifugation and chilled on ice. Anti-mouse immunoresin was coupled to anti-His

antibody for 2 hours at 4 °C, then the immunoresins were washed once with P150T, P500T and P1000, then twice with P20 buffer. Washed immunoresins were mixed with 30-50 µl RRL reaction and stirred on ice for 1.5 hours. Alternatively, immunoresins were mixed with 300 µl from transfected cell lysate and rocked at 4 °C for same time. Then the immunopellets were washed 5 times with buffer containing 10 mM PIPES (pH 7.0), 0.1% Tween-20, and indicated concentration of NaCl. Finally, samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF), and Western blotted as indicated.

3.3 Results

The Physical Interaction between 15-Lipoxygenase-1 (15-LOX-1) and Hsp90. Previous pulldown assays with Hsp90 and Cdc37 antibodies suggested that 15-LOX-1 strongly associates with Hsp90 (Shao, Hartson and Matts, unpublished). To confirm this observation that 15-LOX-1 is an Hsp90-binding protein, His-LOX-1 was expressed in RRL, was immunoadsorbed and immunoadsorptions were Western blotted with an anti-Hsp90 antibody and analyzed by autoradiography. As shown in Figure 14, endogenous rabbit Hsp90 co-immunoadsorbed with human His-LOX-1. The Hsp90 is co-adsorbed immunospecifically compared to the control with the same anti-His co-immunoadsorption, but lacking His-LOX-1 DNA (Figure 14).

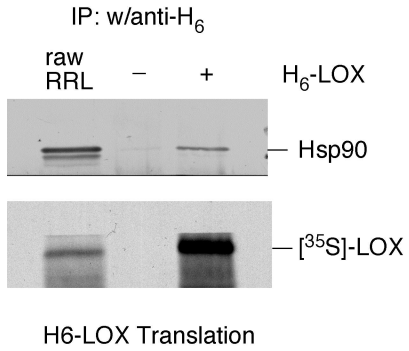


Figure 14: Interaction of endogenous Hsp90 with newly synthesized 15-LOX-1. His-tagged LOX-1 was synthesized in rabbit reticulocyte lysate and immunoadsorbed with anti-His antibody, as described in Materials and Methods. The adsorption was analyzed by Western blotting with anti-Hsp90 (top panel), and auto-radiography (bottom panel). (+) Indicated sample programmed with His-LOX-1 plasmid. The control for non-specific binding was native reticulocyte lysate containing no DNA template (-).

To validate the interaction between Hsp90 and LOX1, another experiment was performed in a human cell line by using transfection. Un-tagged full length 15-LOX-1 plasmid was transfected into K562 myeloid leukemia cells. The cell lysates were immunoadsorbed. The immunoadsorptions were analyzed by SDS-PAGE and Western blot. Again, the result showed that Hsp90 was present in co-immunoadsorption of 15-LOX-1 (Figure 15). This co-adsorption of Hsp90 with His-LOX-1 was specific because Hsp90 was not detected in control lysates from cells that were missing 15-LOX-1 DNA template. This supported our hypothesis that 15-LOX-1 physically associates with Hsp90

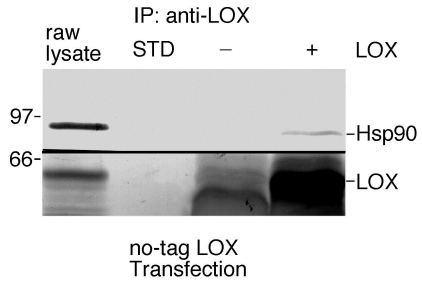


Figure 15: Complex formation between His-LOX-1 and Hsp90 via transfection. Plasmid with no-tagged 15-LOX-1 (+) was transfected into K562 cells. The cells were cultured after 48 hours and the cell pellets were lysed as described in Materials and Methods. Equal amounts of lysates were immunoadsorbed. The immunoadsorptions were then separated by SDS-PAGE. Hsp90 as identified by blotting with anti-Hsp90. (+) Indicated sample programmed with no-tagged LOX-1 plasmid. (-) indicated a negative control from cells that were not transfected. Migrations of molecular weight markers (STD) were indicated (left).

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The Effect of Molybdate on the Hsp90 and 15-LOX-1 Complex in RRL. The biochemical properties of molybdate-bound Hsp90 suggest that Hsp90 is in its "closed" conformation in the presence of molybdate, i.e., molybdate stabilizes interaction of Hsp90 with the client proteins by locking Hsp90 in its clamped conformation (*72*). This high-affinity conformation is created by ATP hydrolysis, and causes Hsp90 and client proteins to form a highly salt-resistant complex (*72*). However, the native complexes between Hsp90 and its chaperones are generally salt-labile (*47*). To characterize whether the relationship between LOX1 and Hsp90 is a client versus chaperone, we tested the effect of molybdate on the association between Hsp90 and 15-LOX-1. His-LOX-1 was synthesized in rabbit reticulocyte lysate. And the lysates were immunoadsorbed. As shown in Figure 16, Hsp90 band was stronger in the presence of molybdate than in the absence of molybdate. This result suggested that Hsp90 and 15-LOX-1 bound in a salt–stable high-affinity complex (Figure 16). This conclusion indicates that *in vitro* 15-LOX-1 behaves like an Hsp90 client, not a partner.

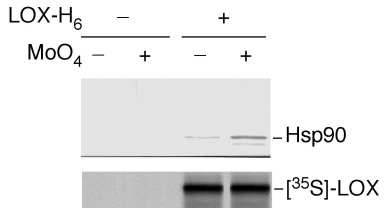

Figure 16: Effect of molybdate on 15-LOX-1. His-tagged LOX-1 protein was synthesized in rabbit reticulocyte lysate via coupled transcription-translation for 30 min at 30 °C. Half of reactions then were treated with 20 mM sodium molybdate for another 5 min. Reactions were chilled, clarified and immunoadsorbed with antibody against affinity tag (anti-His). Immunoadsorptions were washed three times with buffer containing 500 mM NaCl and 20 mM sodium molybdate, and twice with buffer containing 50 mM NaCl and 20 mM molybdate. Washed immunopellets were Western blotted with anti-Hsp90 (top panel, Hsp90) and analyzed by radiography (bottom panel, [^{35}S]-LOX). (+) Indicated sample programmed with His-LOX-1 plasmid. No DNA template as control for non-specific binding (-).

Impact of Geldanamycin on 15-LOX-1 Level in vivo. Hsp90-dependent client proteins become structurally and functionally unstable in the presence of geldanamycin. Thus, geldanamycin causes client protein degradation *in vivo* via many proteolytic pathways. Since our results suggested that 15-LOX-1 was an Hsp90 client protein *in vitro*, we hypothesized that inhibition of Hsp90 function with geldanamycin would impact the 15-LOX-1 level *in vivo.* To test this hypothesis, K562 cells were treated with geldanamycin, and 15-LOX-1 level was analysed via Western blotting. Contrary to our prediction, there was no major difference in the presence or absence of geldanamycin: 15-LOX-1 levels remained stable (Figure 17). This data suggest that 15-LOX-1 is not an Hsp90 client protein.

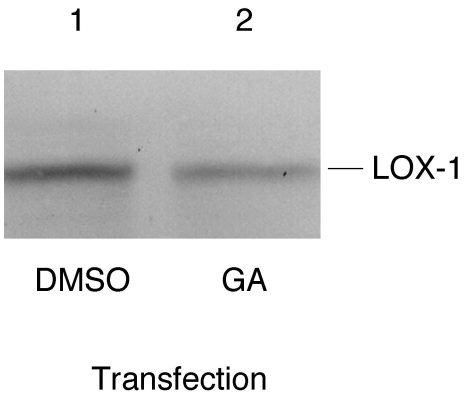

Figure 17: Impact of GA on 15-LOX-1 level in living cells. 15-LOX-1 was transfected into K562 cells. K562 cells were cultured in the presence of 0.1 μM GA and equal volume of DMSO for 24 hours and probed with anti-LOX-1.

3.4 Discussion

The results presented here from these characterizations suggest that 15-Lipoxygenase-1 is a novel Hsp90-binding protein. Physically, 15-LOX-1 strongly associates with Hsp90. 15-LOX-1 strongly co-immunoadsorbs endogenous Hsp90 from rabbit reticulocyte lysate containing His-LOX-1 (Figure 14). Transfection assays (Figure 15) showed that antibody against 15-LOX-1 also co-immunoadsorbs Hsp90 from K562 cell lysates *in vivo*. These data show evidence that the interaction between 15-LOX-1 and Hsp90 is in mammalian cell lysates.

Functionally, however, the data presented here do not support designating 15-LOX-1 as an Hsp90 client. Classic Hsp90 client proteins are degraded *in vivo* via inhibition of Hsp90 by geldanamycin (*2*). In contrast, 15-LOX-1 is strongly stable in molybdate-treated cells (Figure 16). This result demonstrates that 15-LOX-1 does not act like an Hsp90 client *in vivo*, i.e. maturation of nascent 15-LOX-1 or maintaining the

stability of mature 15-LOX-1 protein does not require the Hsp90 help. This conclusion should have a positive control to compare it. Even though we tried to use several known client's antibodies such as Cdk2 and c-Ab1, the experiments did not detect these proteins in untreated cells. I also tried to transfect LOX in Jurket cell and use Lck to be control, but no LOX was expressed. The possible reason is that LOX is toxic, so it is difficult to transfect in Jurkat cell.

Interestingly, even though geldanamycin has no significant effect on 15-LOX-1 *in vivo*, 15-LOX-1 behaves as a client protein in a binding assay (Figure 16). Previous studies show that molybdate causes very strong and highly salt-resistant binding between Hsp90 and client (*47*), Thus, molybdate locks Hsp90 conformation. For 15-LOX-1, molybdate enhances the association between Hsp90 and 15-LOX-1. Thus, the effect of molybdate indicates that 15-LOX-1 acts like a client protein in vitro.

Taken together, these data suggest that 15-LOX-1 binding to Hsp90 is novel. That Hsp90 binding responded to the effect of molybdate suggests that 15-LOX-1 behaves as a client, but the fact that LOX was not degraded by geldanamycin treatment *in vivo* indicates that 15-LOX-1 acts as a co-chaperone. So far, no Hsp90 client protein has been described that behaves like 15-LOX-1.

If 15-LOX-1 is an Hsp90 client protein *in vivo*, there may be two explanations. One is that 15-LOX is a novel Hsp90 client which responses differently to molybdate compared to classic clients. 15-LOX-1 might not follow the known clients' rule that response to the effect of molybdate. Another is that 15-LOX-1 is an Hsp90 client and it degrades in geldanamycin-treated cell. However the rate of 15-LOX-1's degradation may be insignificant. Unlike Lck kinase, 15-LOX-1 might show no dramatic effect in GA-

treated cell *in vivo*, instead only have subtle alteration. This needs pulse-chase experiment to further confirm.

15-LOX-1 might be not an Hsp90 client. Probably it is only an Hsp90-binding protein, i.e., 15-LOX-1 is not an Hsp90 client or Hsp90 co-chaperone. 15-LOX-1 binding to Hsp90 may result from an antibody artifact. But this possibility is unlike since my data do not support.

15-LOX-1 also might be an Hsp90 co-chaperone, but this possibility is unlikely. 15-LOX-1 has no similar structure of known co-chaperone, such as TPR domain. And its behavior also does not act like a co-chaperone.

CHAPTER IV

VCP Interaction With Cdc37

4.1 Introduction

Hsp90 collaborates with co-chaperones to promote folding, functional maturation and stability of client proteins (*2*). Cdc37 is an Hsp90 co-chaperone and was first identified as a 58-kDa product in a mutant strain of *S. cerevisiae* with cell cycle phenotype (*44*). Subsequently Cdc37 has been revealed to play a critical role in the targeting of Hsp90 to client protein kinase. Hence it is called "kinase specific co-chaperone" (*83*). Furthermore, Cdc37 may also be a chaperone independent of Hsp90 and may facilitate folding and stabilization of protein kinases (*48*).

Valosin-containing protein (VCP) is an AAA+ (ATPase associated with diverse cellular activities) protein, and one of the most abundant intracellular proteins. It plays a critical function in assisting membrane tracking, organelle biogenesis and protein degradation (*84-86*). Prince et al. showed that VCP, a novel Hsp90 co-chaperone, might be in the same heterocomplexes as Hsp90, and might facilitate them to fold kinase protein properly (*87*). The data presented in this report suggest that the interaction between VCP and Cdc37 is uncertain.

4.2 Materials and Methods

Reagents. K562 and Jurkat cells were obtained from ATCC. Mouse VCP antibody was purchased from Affinity BioReagents (catalog#: MA3-004). Monoclonal mouse Cdc37 antibody was also from Affinity BioReagents (catalog#: MA3-029). The JJ3 monoclonal antibody against p23 was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN)). Polyclonal mouse antibody to Hsp90 and Cdc37 were described previously (*47*). Irrelevant control antibody (MOPC-21) was purchased from Sigma (M7894). Nuclease-treated rabbit reticulocyte lysate (RRL) was from Promega. NHS-activated Sepharose^{TM} 4 Fast Flow was purchased from Amersham Biosciences. Alkaline phosphatase-conjugated affinity rabbit anti-mouse IgG antibodies were from Jackson ImmunoResearch.

4.3 Results

In the immunoadsorptions using polyclonal and monoclonal anti-Cdc37 antibody, a protein that migrates more slowly than Hsp90 is observed on one-dimensional electrophoresis gels. Peptide mass figureprinting identified that this protein is VCP, an AAA+ protein (Shao, Matts and Hartson, unpublished). Consistent with this observation, the work of Prince et al. suggests that VCP might be an Hsp90 co-chaperone partner (*87*). Thus, we hypothesized that VCP might interact with Cdc37 and might play a role in recognizing tyrosine kinases for kinase regulation or localization.

Co-immunoadsorption with Endogenous Complexes in Lysate of Cultured Jurkat T-cell. To test our hypothesis**, t**hree different kinds of anti-Cdc37 antibodies were utilized to co-immunoadsorb VCP. Polyclonal Cdc37 antibody specifically co-adsorbed VCP

compared to the control lysate from the Jurkat cells but lacking the DNA template (Figure 18A). In contrast, monoclonal Cdc37 antibody only could capture a small amount of VCP. It was not significant if compared to the control. Even though the amount of monoclonal Cdc37 was less than that of polyclonal Cdc37, the amount of VCP adsorbed with Cdc37 was not proportional to the amount of Cdc37 (Figure 18A). The third antibody: rabbit Cdc37 also could not immuno-specifically capture VCP (Figure 18B). This result suggests that association between VCP and Cdc37 might be caused by cross-reaction or that immuno-adsorption might be prevented by epitope masking or steric hinderence.

To further test the hypothesis that VCP interacts with Hsp90 partner, p23. JJ3 antibody was used to co-immunoadsorb VCP. The JJ3 antibody could catch VCP, but the association between p23 and VCP was weak (Figure 18A). From this result, it is difficult to draw the conclusion that Hsp90 partner, p23 or Cdc37, interacts with VCP.

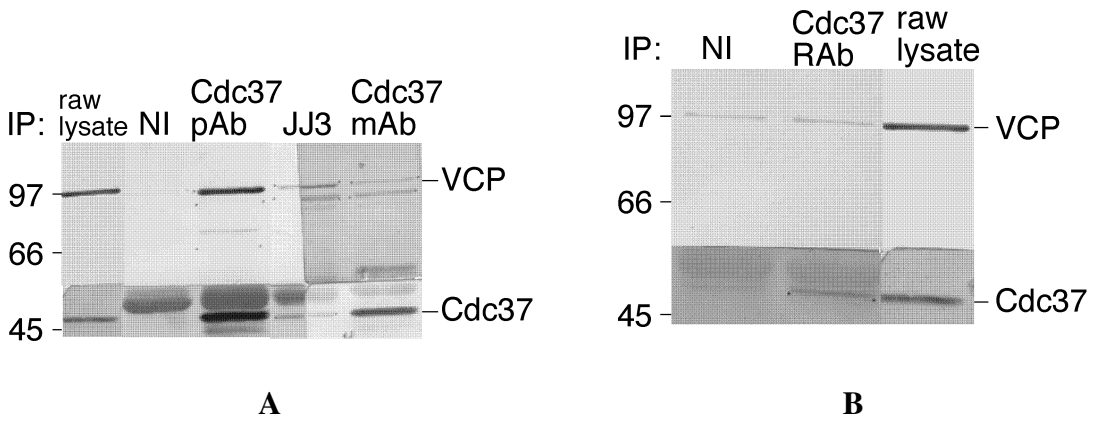

Figure 18: Hsp90 co-chaperone Cdc37 and p23 co-immunoadsorptions from T-cell lysate. (A) Polyclonal Cdc37 (Cdc37pAb), monoclonal Cdc37 (Cdc37mAb) and JJ3 or irrelevant non-immune antibody (NI) were used to immunoadsorb lysates of Jurkat cell. Immunoadsorptions were washed once

with buffer containing 50mM NaCl and three times with buffer containing 100mM NaCl. Adsorptions were analyzed by SDS-PAGE, and Western blotting with anti-VCP (top panel) and anti-Cdc37 (bottom panel). (B) Immunoadsorption with rabbit polyclonal Cdc37 antibody was also probed with anti-VCP and anti-Cdc37. Migrations of molecular weight markers are indicated.

*Co-immunoadsorption with VCP in Rabbit Reticulocyte Lysates. T*o further determinate if the VCP and Cdc37 are in the same complex, immunoadsorptions with monoclonal and polyclonal anti-Cdc37 and JJ3 were performed in rabbit reticulocyte lystate. Again, VCP was readily recovered only in the immunoadsorption with polyclonal anti-Cdc37, but not in the immunoadsorptions with the other two anti-Cdc37 antibodies (Figure 19).

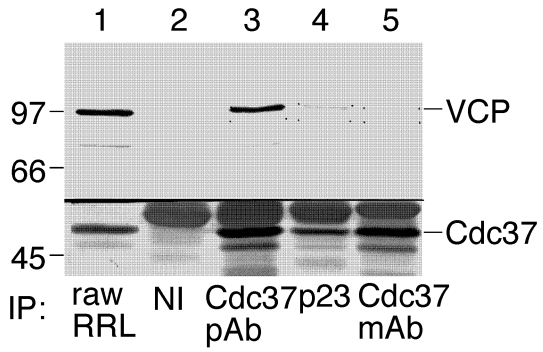

Figure 19: Co-immunoadsorption with anti-Cdc37 and anti-p23 in rabbit reticulocyte lystate. Cdc37 and JJ3 antibodies immunoadsorbed in RRL. The adsorptions were analyzed by Western blotting with anti-VCP (top panel) and anti-Cdc37 (bottom panel). NI denotes immunoadsorption with mouse non-immune antibody. Raw RRL indicates lysates directly from rabbit reticulocyte for positive control. Migrations of molecular weight markers are indicated.

Co-immunoadsorption with Epitope-tagged Complexes in K562 Cell and RRL. According to the above results that only polyclonal Cdc37 can capture VCP, it is possible

that the recognition of VCP is result from Cdc37 antibody cross-reaction. To address this possibility, antibody against His affinity-tag was used to immunoadsorb via coupled transcription-translation in RRL. Affinity His tagged antibody was also used in control lysates from cells that were lacking DNA template. Consistent with hypothesis that the association between VCP and Cdc37 is antibody cross-reaction, the result showed that immunoadsorption with anti-His can capture His-tag Cdc37, but it did not co-adsorb VCP (Figure 20). This suggests that VCP and Cdc37 might not be in the same complex.

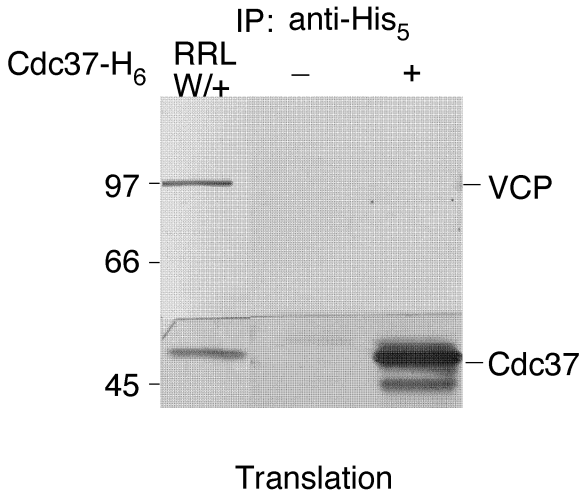

Figure 20: Immunoadsorption epitope-tagged Cdc37 in RRL. His-tagged Cdc37 was synthesized in rabbit reticulocyte lysate using coupled transcription-translation. The immunoadsorption with anti-His antibody was described in Chapter II. The adsorptions were analyzed by Western blotting with anti-VCP (top panel) or anti-Cdc37 (bottom panel). The control for non-specific binding was native reticulocyte lysate containing no DNA template (-). A standard marker for migrations of molecular weight is indicated.

To confirm the above results, we repeated the co-immunoadsorption via transfection. K562 cells were transfected with plasmid DNA encoding affinity tagged Cdc37 (Flag-Cdc37). Then the antibody against the Flag affinity-tag was used for immunoadsorption. After Western blotting the adsorptions, VCP was not detected (Figure

21A). We also performed reciprocal analysis: antibody against the Flag affinity-tag could not co-immunoadsorb Cdc37 from cell lysates containing Flag-VCP (Figure 21B).

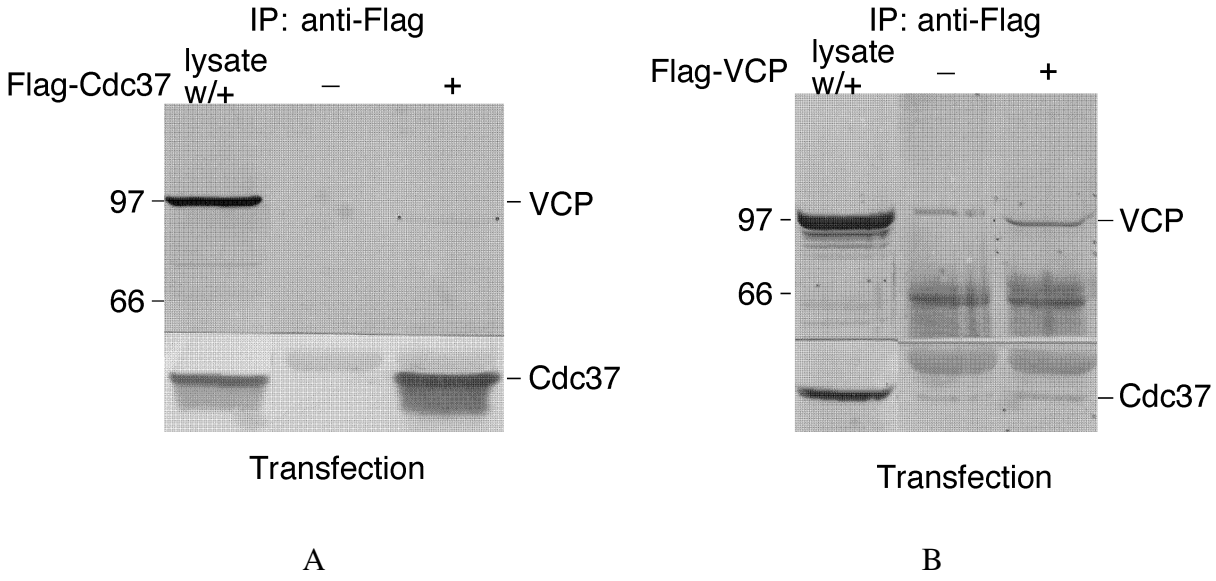

Figure 21: Immunoadsorption of epitope-tagged Cdc37 and VCP in K562. (A) K562 cells were transfected with plasmid encoding affinity-tagged Cdc37 (Flag-Cdc37) or with no DNA template and cultured 40 hours. After culture, lysates were prepared as described in Chapter II, and immunoadsorbed with anti-Flag antibody. Immunoadsorptions were Western blotted with anti-VCP (top panel) and anti-Cdc37 (bottom panel). (B) Reciprocal analysis: plasmid encoding Flag-VCP was transfected into K562 cells, cell pellets were lysed and analyzed by immunoadsorption and Western Blotting.

4.4 Discussion

The results presented above indicate our hypothesis that VCP might interact with Cdc37 is not well supported. The evidence is that only polyclonal Cdc37 can co-absorb the abundant amount of VCP, but the other techniques cannot. Thus, the explanation is that the interaction between VCP and Cdc37 is not real; instead that it is an antibody

cross-reaction which polyclonal Cdc37 antibody recognized VCP and co-immunoadsorbed it directly during pull down assay. Based on the weak data, I made the decision to stop this project.

CHAPTER V

Mini-Chromosome Maintenance (MCM) Interaction With Cdc37

5.1 Introduction

Co-chaperone Cdc37 and Hsp90 associate with kinase proteins to help their proper folding and stabilize their structure (*2*). Hence, Cdc37 is termed as the "kinase specific co-chaperone" (*83*). Cdc37 may also play an important role in acting as a chaperone independent of Hsp90. Recent studies suggest that some endogenous Cdc37 does not associate with Hsp90 (*49*).

Mini-chromosome maintenance (MCM) proteins belong to AAA (associated with various cellular activities) family and display ATPase activity (*88*). They are eukaryotic replication factors required for the initiation of DNA replication (*89*). MCM 2-7 are evolutionally conserved in all eukaryotes (*88, 90*). These MCM proteins form a hexameric complex. This complex is an important element of the prereplication complex. MCM proteins are regulated in conjunction with cell cycle (*91*).

Monoclonal anti-Cdc37 antibody pull-down assay shows that several associated proteins are on one-dimensional electrophoresis gel (Hartson and Te, unpublished). Peptide mass fingerprinting identified these proteins as MCMs. This result raises our hypothesis that Cdc37 might regulate the functions of MCM protein. In this report, the

data presented here imply that MCM does not interact with Cdc37. The association between MCM and Cdc37 is conditionally false.

5.2 Materials and Methods

Reagents. Mouse monoclonal MCM7 antibody (catalog#: sc-9966), rabbit polyclonal Mcm7 (catalog#: sc-22782) and goat polyclonal MCM3 (catalog#: sc-9850) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal Cdc37 antibody was from NeoMarkers (catalog#: RB-039). The JJ3 monoclonal antibody against p23 was a gift from Dr. David Toft (Mayo Clinic, Rochester, MN)). Polyclonal mouse antibody to Hsp90 and Cdc37 were described previously (*47*). Irrelevant control antibody (MOPC-21) was purchased from Sigma (M7894). FKBP52 antibody was a gift from Dr. David Smith. Antibody recognizing poly-His epitope tags was from QIAGEN (catalog 34660). NHS-activated Sepharose^{TM} 4 Fast Flow was purchased from Amersham Biosciences. Alkaline phosphatase-conjugated affinity rabbit anti-mouse, anti-rabbit and anti-goat IgG antibodies were purchased from Jackson ImmunoResearch. K562 and Jurkat cells were obtained from ATCC.

5.3 Results

Co-immunoadsorption with Endogenouse Complexes in T-cell Lysate. To test whether Cdc37 has a physical interaction with MCM proteins, three different kinds of Cdc37 antibodies were used to immunoadsorb Cdc37 and the Cdc37 complexes. Subsequently, MCMs were detected by Western blotting. MCM was readily detectable in immunoadsorptions using mouse monoclonal Cdc37 antibody (Figure 22A) and was

weakly detectable in immunoadsorption using polyclonal Cdc37 (Figure 22B). In contrast to these mouse antibodies, the rabbit polyclonal Cdc37 could not capture MCM (Figure 22C). All three anti-Cdc37 antibodies caught almost equal amount of Cdc37, but they did not co-adsorb similar amount of MCM. This result suggested that the association between MCM and Cdc37 was possibly a monoclonal antibody cross-reaction. Alternatively, this co-immunoadsorption was prevented by epitope masking or steric hindrence.

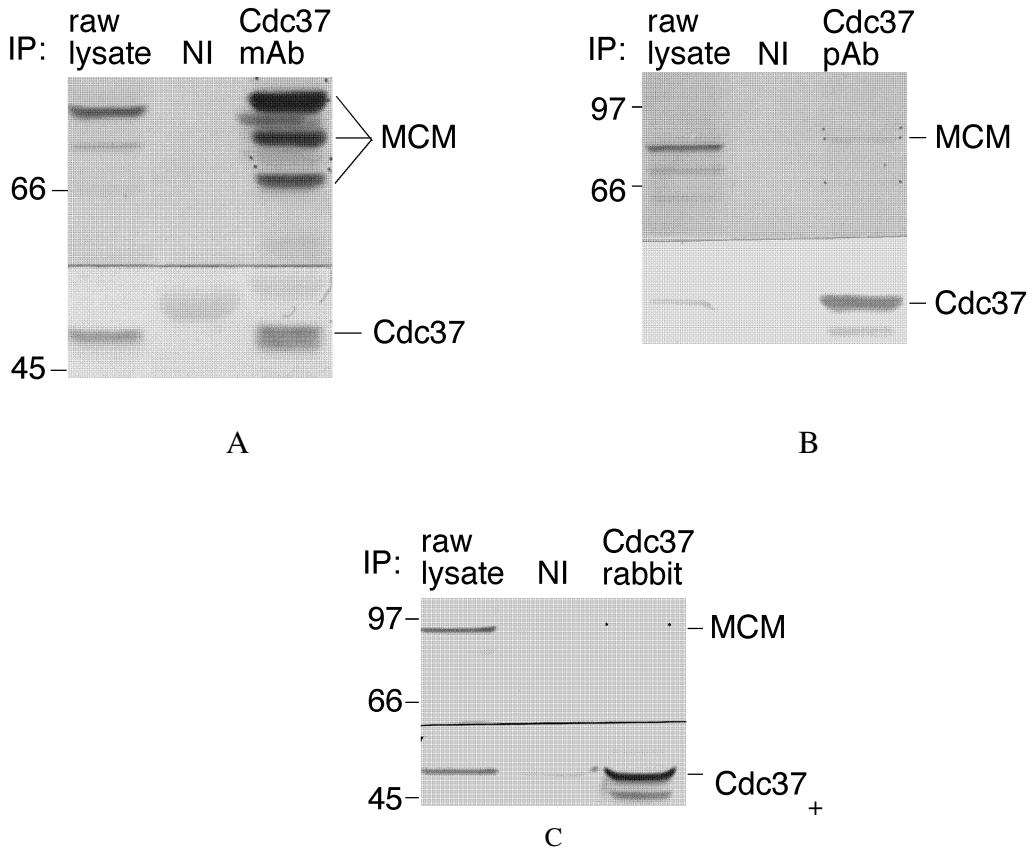

Figure 22: Co-immunoadsorptions with different kinds of Cdc37 antibodies in T-cell lysate. Antibodies against Cdc37 and non-immune antibody were used to immunoadsorb MCM. Immunoadsorptions were washed once with buffer containing 50 mM NaCl and four times with buffer containing 20 mM NaCl. Membranes were probed with anti-MCM (top panel) and anti-Cdc37 (bottom panel). Migrations of molecular weight markers are indicated. (A) Adsorptions with mouse monoclonal

Cdc37 antibody. (B) Adsorptions with mouse polyclonal Cdc37 antibody. (C) Adsorption with rabbit polyclonal Cdc37 antibody.

To further assess the interaction between MCM and Cdc37, we performed the reciprocal analysis: immunoadsorptions using the different antibodies against MCM proteins were subjected to Western blot analysis for co-adsorption of Cdc37. Opposite to our hypothesis, both rabbit and goat anti-MCM did not capture Cdc37 (Figure 23A&B). Thus, reciprocal immunoadsorptions demonstrated that co-immunoadsorptions with two anti-MCM antibodies are not efficient to adsorb Cdc37. Alternatively the recovery of MCM in immunoadsorptions of monoclonal Cdc37 probably was an immunological artifact.

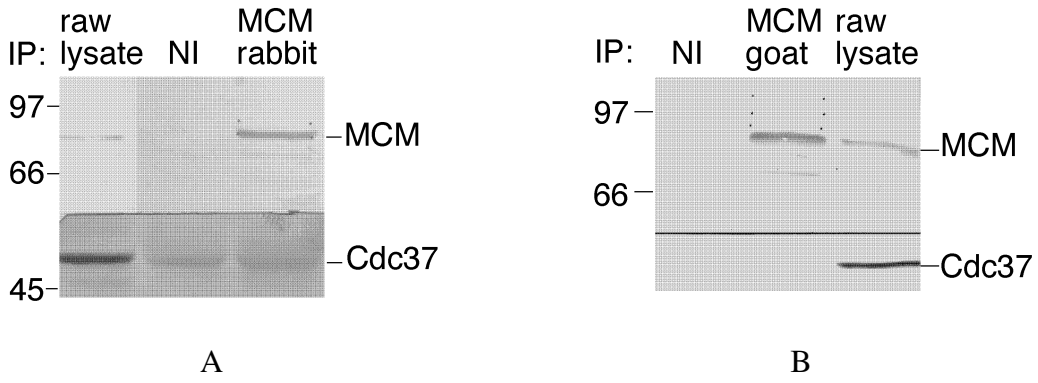

Figure 23: Co-immunoadsorptions with two different anti-MCM antibodies. Antibodies against MCM and non-immune antibodies were used to adsorb. Immunoadsorptions were washed once with buffer containing 50mM NaCl and four times with buffer containing 20mM NaCl and then were identified by probing with anti-MCM (top panel) and anti-Cdc37 (bottom panel). Migrations of molecular weight markers are indicated. (A) Adsorption with rabbit polyclonal MCM antibody. (B) Immunoadsorption with goat polyclonal MCM antibody.

To test whether MCM interacts with other Hsp90 co-chaperones, two other Hsp90 partner antibodies (anti-FKBP52 and JJ3) were used to immunoadsorb lysates of cultured Jurkat T-cell. Again, immunoadsorption with anti-FKBP52 antibody did not catch MCM, but it could catch small amount of Cdc37 (Figure 24A). Similar to FKBP52, absorption with JJ3 antibody could capture cdc37, but not MCM (Figure 24B). This result indicated that MCM did not interact with Hsp90 co-chaperones (FKBP52 and p23).

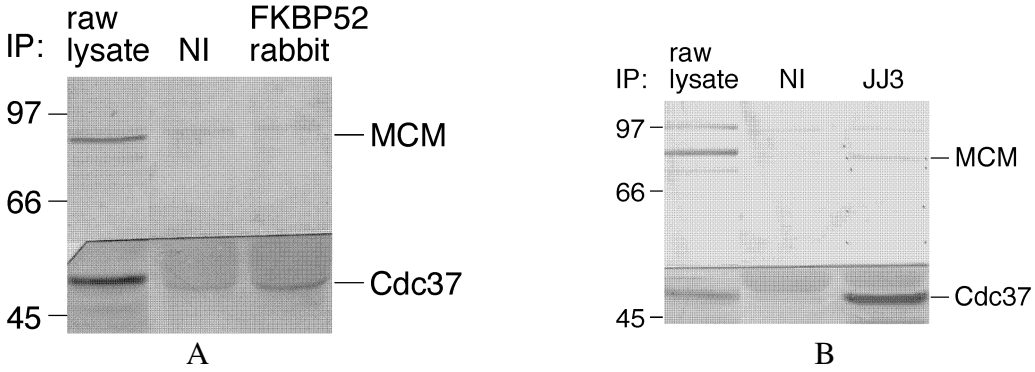

Figure 24: Co-immunoadsorptions with Hsp90 co-chaperones (FKBP52 and JJ3). T-cell lysates were subjected to co-immunoadsorption with antibodies and non-immune antibody (NI). Immunoasorptions were washed four times with buffer containing 50 mM NaCl and then probed with anti-MCM (top panel) and anti-Cdc37 (bottom panel). Migrations of molecular weight markers are indicated. (A) Adsorptions with anti-FKBP52 antibody (FKBP52 rabbit). (B) Adsorptions with JJ3 antibody (p23).

Co-immunoadsorption with Epitope-tagged Complexes in K562 Cell and RRL. To further assess the hypothetical interaction of MCM and Cdc37, His-tagged Cdc37 was translated in RRL, adsorbed, and adsorptions were assayed by Western blotting for anti-MCM. The data suggested that MCM weakly associated with Cdc37 in RRL (Figure 25).

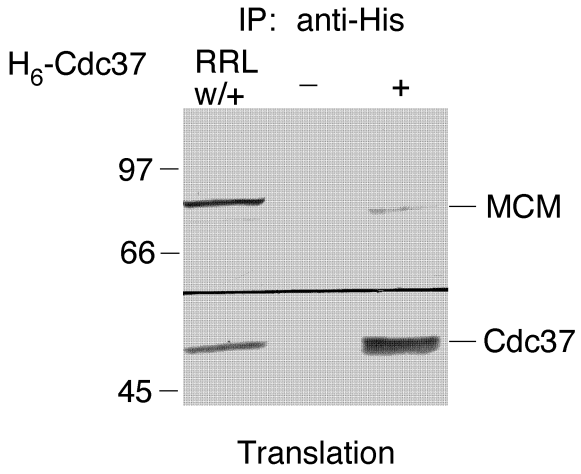

Figure 25: Co-immunoadsorption with epitope-tagged Cdc37 in RRL. His-tagged Cdc37 was synthesized in rabbit reticulocyte lysate and adsorbed with anti-His antibody. The immunoadsorptions were analyzed by Western blotting with anti-MCM (top panel) or anti-Cdc37 (bottom panel). The control for non-specific binding was immunoadsorbed native reticulocyte lysate containing no DNA template (-). Migrations of molecular weight standards are indicated. RRL w/+ indicates RRL containing translated His-tagged Cdc37.

We also performed co-adsorption with anti-MCM antibody using *in vivo* transfection. K562 cells were transfected with plasmid DNA encoding Flag-tagged Cdc37. Flag-Cdc37 was adsorbed using antibody against the Flag affinity tag. Similar as before, MCM was not detected to co-adsorb with Flag-Cdc37 (data not shown).

5.4 Discussion

According to above summary, our hypothesis that Cdc37 interacts with MCM is conditionally false. In all experiments, only mouse monoclonal Cdc37 antibody could strongly and reproducibly capture MCM. Mouse polyclonal Cdc37 and His-Cdc37 could capture a little bit of MCM, but the rabbit polyclonal Cdc37 could not catch MCM. Furthermore, neither of two different kinds of MCM antibody (rabbit and goat) nor Flag-

Cdc37 could co-immunoadsorb MCM at all. Therefore, monoclonal Cdc37 antibody may cross-react with MCM. Another possibility is epitope masking. This explanation is unlikely because MCM also could not be caught by co-immunoadsorptions with several different epitope's antibodies. Compared to co-adsorptions between monoclonal, polyclonal and rabbit Cdc37, almost equal amount of Cdc37 can be caught, but Cdc37 cannot co-adsorb equal amount of MCM. Thus, it is impossible to draw the conclusion that difficultly catching MCM might result from low amount of Cdc37 recovery.

However recent studies indicate that MCM has been identified to be an Hsp90 co-chaperone in yeast (*74*). Therefore, the interaction between Cdc37 and MCM could be real. Nonetheless, the data demonstrate that interaction between MCM and CDC37 is uncertain.

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Scope and Method of Study: The purpose of this study was to confirm identify of Armadillo Repeat Chaperone Binding Protein 2 (ARCBP2) and interaction with ARCBP2 and Hsp90; to characterize 15-lipoxygenase-1 (15-LOX-1) interaction with Hsp90. Pull-down assays, Western blotting and Mass spectrum fingerprinting assay determined the association between Hsp90 and proteins. The mechanism of inhibition of Hsp90 by drug geldanamycin and molybdate *in vitro* was studied using coupled transcription/translation in nuclease reticulocyte lysate, and was analyzed using pull-down assays, Western blotting and autoradiography. Hsp90-dependent signaling pathway were examined in K562 cell using transfection by treating with drug geldanamycin, followed by immunoadsorption and Western blotting

Findings and Conclusions: ARCBP2 associates with Hsp90 and it acts as a novel Hsp90 co-chaperone partner. 15-lipoxygenase-1 physically interacts with Hsp90. But it is still mystery for being an Hsp90 client.

ADVISER'S APPROVAL: Dr. Steven D. Hartson