DEFINED INTERACTIONS OF PROTEIN KINASES

WITH HSP90 AND CDC37

By

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PREFACE

Protein kinases function to transduce the countless number of signals that allow a cell to grow and adapt to its environment. A significant number of protein kinases require assistance in being folded and converted into functional signal transducers from nascent polypeptides or in maintaining their functional conformation once folded. This assistance is provided by the molecular chaperone Hsp90 and its cohort Cdc37 which together function to fold protein kinases through a series of direct interactions. A dynamic process which remains poorly understood.

In this work the interactions of three unique protein kinases with Hsp90 and Cdc37 were studied. Lck, a Src-family tyrosine kinase, which is widely known to interact with Hsp90 and Cdc37. Cdk2, a cyclin-dependent protein kinase, found to genetically although not physically interact with Hsp90 and Cdc37. And JNK1α1, a stress activated protein kinase, which has shown not to interact with either Hsp90 or Cdc37. To study these three kinases deletion mutagenesis and co-immunoabsorption techniques were primarily used to determine the molecular interactions of endogenous Hsp90 and Cdc37 with each newly synthesized protein kinase construct. The results of this work indicate that: (i) Hsp90 interacts with structural motifs found in both the N-terminal and C-terminal lobes protein kinases; (ii) Cdc37 interacts exclusively with N-terminal lobe of protein kinases; (iii) depending on the kinase the presence of different structural motifs within the N-terminal lobe are required for Cdc37 interaction; and (iv)

the minimum protein kinase structure required for high affinity salt stable Hsp90 and Cdc37 interaction is the N-terminal lobe through the stabilized αE-helix in the C-terminal lobe. In turn, these findings provide insight into how Hsp90 and Cdc37 recognize, bind and fold client protein kinases. In addition, these findings provide clues to the evolutionary relationship between the Hsp90 chaperone machine and protein kinases.

In appreciation of their contributions to this work and my overall development I would like to thank the following: Dr. Robert L. Matts for his relentless imagination, optimism, and patience. Dr. Steven D. Hartson for his grounded and steadfast approach to science and the countless number of pep talks. My lab mates Liang Sun, Dr. Jieya Shao, Dr. Bo-Geon Yun, Dr. Bradley T. Scroggins, Jessica Matts, Yanwen Guo, Dr. Sheri Uma, Angela Irwin, and Wenjun Huang for their insightful and sometimes entertaining contributions. Janet Rogers, SueAnn Hudiburg and Kate Cooley for their precision work in the core facility. My roommates James McDoulett and Crystal Power for giving me a place to live. My friend Audrey Vecoven just because. My committee Dr. Richard C. Essenberg, Dr. Jose L. Soulages, Dr. Michael Massiah, and Dr. Robert Burnap. My father and step-mother Dan and Kristi Prince for their support. My caring sister Jessica Prince. My brother Michael Prince for his confidence and encouragement. My grandmother Mary Lee Ruther for believing in me. And most of all, I would like to dedicate this work and what ever may come of it to my late mother Suzanne I. Prince, who always wanted more for her children.

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NOMENCLATURE

17-AAG	17-allylamino-17-demethoxygeldanamycin
ADP	adenosine diphosphate
Aha I	activator of Hsp90 ATPase activity
Akt 1	also known as PKB (protein kinase B)
APH(3')-IIIa	aminoglycoside phosphotransferase
ATP	adenosine triphosphate
AU5-tag	peptide-TDFYLK
Bag	Bcl-2 (B-cell lymphoma gene 2) associated gene product
BLM	Cdc37 Bag-like motif
CAK	Cdk activating kinase
CD	Hsp90 C-terminal Domain
Cdc	cell cycle dependent component
Cdk	cyclin dependent kinase
CHaK	transient receptor potentinal channel kinase
CITD	C-terminal inhibitory tail domain
CKII	casein kinase II
CL	protein kinase C-terminal lobe
Csk	c-Src kinase
CSM	protein kinase non-catalytic C-terminal structural motif
DMSO	dimethyl sulfoxide
ErbB2	epidermal growth factor receptor B2
Fkbp	FK506 binding protein
Flag-tag	pepetide-DYKDDDDK
GA	geldanamycin
GHKL	DNA gyrase histidine kinase ligase protein family
GR	glucocorticoid receptor
Grp	glucose-regulated protein
Harc	Hsp90-associating relative of Cdc37
HBD	Cdc37 Hsp90 binding domain
Hck	hemopoietic cell kinase
Hip	Hsp70 interacting protein
His-tag	peptide-HHHHHH
Hop	Hsp70/Hsp90 organizing protein
HRI	heme-regulated inhibitor of protein synthesis eIF2α kinase
Hsc	heat shock cognate protein
Hsp	heat shock protein
HtpG	high temperature protein G

JNK	c-Jun N-terminal kinase
KBD	Cdc37 kinase binding domain
LBD	ligand binding domain
Lck	lymphoid cell kinase
LD	Hsp90 linker domain
MAPK	mitogen activated protein kinase
MD	Hsp90 middle domain
NAD	Hsp90 N-terminal ATPase domain
NCSM	protein kinase non-catalytic structural motifs
NDM	NAD dimerization motif
NL	protein kinase N-terminal lobe
NLS	nuclear localization signal
NSM	protein kinase non-catalytic N-terminal structural motif
p23	23 kDa protein
PDB	Protein Database www.rcsb.org
PDK1	phosphoinositide-dependent kinase
PP5	protein phosphatase 5
PSTK	serine/threonine protein kinase
PYK	tyrosine protein kinase
Raf1	Ras activated factor kinase
Rio2	ribosome processing kinase 2
SAPK	stress activated protein kinase
SH2	Src-homology domain 2
SH3	Src-homology domain 3
SHR	steroid hormone receptor
Src	rous sarcoma oncogene protein kinase
TnT	coupled transcription and translation
TPR	tetratricopeptide repeat
Trap1	type-1 tumor necrosis factor receptor associated protein
TTC4	tetratricopeptide repeat containing protein 4
TRiC	tailless complex polypeptide 1 (TCP-1) ring complex

CHAPTER I

Introduction and Background

Proteins are the molecular machines that give all biological entities the ability to exist and adapt effectively to their environment. The central process in biology is the conversion of information encoded by genetic material into macromolecular units capable of functioning as biological machines. Therefore, the synthesis of a protein can simply be described as the translation of one linear polymer composed of four different nucleic acid bases into another linear polymer composed of twenty different amino acid residues by a machine made up of both amino acids and nucleic acids. Additionally, in order for this newly-synthesized amino acid polymer to become a functional biological component it must fold and convert from a linear polypeptide chain into a three-dimensional tertiary structure.

The process of a protein folding into its native structure is directed along one or more specific free energy pathways in which each conformation is specifically restricted by a set of steric, attractive, and repulsive forces. This is opposed to the protein randomly sampling each possible conformation (*1, 2*). Along these lines, one of the principle forces that direct protein folding is the coordinated removal of hydrophobic amino acid side chains from the surrounding aqueous environment, in order to nucleate a hydrophobic core. This eventually allows the rest of the amino acid residues to assemble the secondary structures and tertiary interfaces that collapse the protein into its native conformation (*2*).

According to the Anfinsen theory, the ability of a protein to fold into its structure is an inherent property of its primary amino acid sequence, based on observations that small denatured proteins are capable of spontaneously re-folding back into their native conformation *in vitro* (*3*). However, *in vivo* the ability of a nascent polypeptide to spontaneously fold into the correct conformation is much more difficult due to the cell's congested and dynamic environment which has an estimated protein concentration of 300 mg/ml (*4*). This environment creates a competition between the intramolecular interactions with the protein itself and the intermolecular interactions with the surrounding proteins. Thus, as a polypeptide folds, other proteins are able to interact with it and possibly prevent it from folding into its native conformation. This can then lead to aggregation of the polypeptide with other mis-folded proteins, which is often harmful to the cell. This potential for aggregation is especially prevalent for proteins with multiple domains which must be properly oriented within the protein itself, in order to remain stable and functional (*5*).

To prevent possible mis-folding of polypeptides, cells have developed a number of efficient mechanisms that assist proteins in proper folding. The components that mediate these mechanisms are proteins themselves and are referred to as molecular chaperones, due to their ability to assist in folding polypeptides into their correct conformation without remaining associated with the final folded protein product. Thus in as sense, molecular chaperones play a role critical *in vivo* in maintaining the conversion of genetic information into the functional molecular machines needed to sustain life (reviewed in (*6-8*)).

Molecular chaperones are highly conserved proteins that are present in all cell types, and are constitutively and inducibly expressed. Because the expression levels of a number of chaperones are elevated during times of heat shock or glucose-deprivation, the designations heat shock protein (Hsp) and glucose-regulated protein (Grp) arose. Subsequently, expression of these proteins was found to be inducible by other cellular stresses. In the cytoplasm, chaperones can bind directly to the ribosome, such as the peptidyl-prolyl isomerase trigger factor (TF) in *Escherichia coli*, and the 70 kDa heat shock protein (Hsp70) family members Ssb1 and Ssb2 and the ribosome associating complex (RAC) in *Saccharomyces cerevisiae*. Other members of the Hsp70 family (DnaK homologues in bacteria) along with their cohorts help fold newly synthesized polypeptides and direct them to larger multimeric chaperonins, such as the GroEL ring complex in bacteria or the TRiC ring complex in eukaryotic cells. Nascent polypeptides can also be folded and directed to the TRiC complex by the GimC/prefoldin chaperones which are found in both archea and eukaryotic cells, but not bacteria. Also, working in concert with Hsp70, is the molecular chaperone Hsp90, which has been the focus of a large number of studies, including this one due to its specific and unique set of substrates or "client proteins" that it chaperones (reviewed in (*6, 9-11*)).

Hsp90

Hsp90 is a vital ATPase driven molecular chaperone that is required for viability in all eukaryotic cells. Expressed at high levels, Hsp90 consists of 1-2% of the total cytosolic protein in a cell and during times of stress, expression can rise above 4% (*12, 13*). This level of expression reflects the role of Hsp90 not only in folding, but also in

maintaining the activity and stability of a wide variety of specific proteins. Though, the method through which Hsp90 recognizes such a diverse, yet explicit set of protein clients is not understood.

Many of Hsp90's client proteins are responsible for the transduction of the signals that cue a cell to grow, divide, differentiate, die or simply adjust to its dynamic environment. For this reason Hsp90 is often referred to as the "signal transduction chaperone". Client proteins include steroid hormone receptors, transcription factors, and protein kinases, among others (Table 1). However, Hsp90 and its cohorts do not only aid in the biogenesis of these client proteins, but also help regulate their activity by maintaining them in an inactive, yet activatable conformation. For example, Hsp90 is required to maintain steroid hormone receptors in a conformation that can efficiently bind ligand, or in the case of other clients, to be post-translationally modified or activated by some other protein-protein interaction (reviewed in(*9, 14, 15*)). This ability to fold and regulate these signal transduction proteins places Hsp90 at the crossroads of most all signal transduction pathways. Although only a few members of a signaling pathway may actually interact with Hsp90, as is often seen in protein kinase cascades, the maintenance of these few members is crucial for the integrity of the entire signaling pathway. In addition, Hsp90 has been shown to be able to act as evolutionary buffer that can support the conformational diversity of signal transduction proteins that have undergone mutational drift, thereby at times masking genotypic differences (*16-19*) This may be especially important in the development of certain transformed cell populations, as Hsp90 seems to support the function of numerous proto-oncogenic client proteins (*20-23*).

The ability of Hsp90 to support these aberrant cell populations makes it a promising target for cancer therapy. As it is, the benzoquinone ansamycin derivative 17-AAG (17-allylamino-17-demethoxygeldanamycin), a potent inhibitor of Hsp90's ATPase activity, was the first Hsp90-inhibitor to reach clinical trials for the treatment of various cancers (*24, 25*). Currently, there are 26 ongoing cancer trials in various phases based on Hsp90 inhibition (*24-27*).

Hsp90 Relatives

In eukaryotic cells, Hsp90 exists primarily in the cytoplasm with family members also found the endoplasmic reticulum, mitochondria and chloroplast. Prokaryotes also have an Hsp90 homologue called HtpG (*28*), which is most likely the original version of the molecular chaperone. However, unlike eukaryotic Hsp90, HtpG is not required for viability under normal growing conditions. In mammalian cells, two genes code for cytosolic Hsp90 with human Hsp90α having 85% sequence identity to human Hsp90β. Across evolution Hsp90 is also highly conserved with human Hsp90α being 60% identical to yeast Hsp90 and 40% identical to *E. coli* HtpG.

One of the major differences between the two mammalian genes is their expression levels. Hsp90α is induced upon heat shock and other stresses, whereas Hsp90β seems to be constitutively expressed and is sometimes referred to as Hsc90 (*29, 30*). The enigmatic Hsp90 family member responsible for the chaperoning of proteins that transverse the endoplasmic reticulum is known as Grp94 (94 kDa glucose-regulated protein) (*31*). In humans, Grp94 is 46% identical to Hsp90α, and has two unique N-terminal and C-terminal extensions. The remaining mammalian Hsp90 family member,

Trap1 (tumor necrosis factor receptor associated protein type 1) or Hsp75 is 35% identical to Hsp90β and resides primarily in the mitochondria (*32*), despite its name which suggests a more cytosolic function. Trap1 has also been found to bind the retinoblastoma protein in the cytosol (*33*).

Hsp90 Protein Structure

Functional Hsp90 exists as either a homodimer (α–α, β–β) or a heterodimer (α–β) with its quaternary structure dictated by its ATPase activity (*34-36*). Each Hsp90 monomer consists of four defined domains: an N-terminal ATPase domain (NAD), a charged linker domain (LD), a middle domain (MD), and a C-terminal dimerization domain (CD) (Fig. 1 and Fig.2).

The NAD is the most conserved domain of Hsp90 with its ATP binding cleft roughly spanning residues 1-220. X-ray crystal structures of the both yeast and human N-terminal domain of Hsp90 have been solved and show that it consists of a series of exposed curved anti-parallel β-sheets on one side meshed against a series of α-helices, with the ATP binding pocket found between the β-sheets and the α-helices (*37-39*). This pocket is also the site where the Hsp90 inhibitor geldanamycin (GA) binds, a benzoquinone ansamycin antibiotic which induces Hsp90 to adopt the ADP bound state (*40-42*).

The unusual fold of the N-terminal domain of Hsp90 is known as a Bergerat fold, and is similar to other ATPases belonging to the GHKL family, which consists of DNA gyrases, helicases, and histidine kinases (*43, 44*). In each protein, ATP binding, hydrolysis, and exchange is utilized to drive the conformational changes that confer their

activity, with ATP binding in Hsp90 causing the dimerization of the NAD. This is coupled with the constitutive dimerization of the CD, which results in a ring-like structure, which is often referred to as the "molecular clamp" (*45*). This ATP-bound conformation with the client results in its tight interaction with Hsp90, which is also referred to as the "slow on/slow off" conformation. In contrast, the ADP-bound conformation of Hsp90 is referred to as the "fast on/fast off" conformation due to its weak interaction with the client protein (*46*).

As alluded to earlier, the ADP-bound conformation can be mimicked by the binding of GA to the nucleotide binding cleft, thus disrupting the ability of Hsp90 to bind ATP and dimerize into its "molecular clamp"(*37*). This results in the disruption or weakening of the interaction between Hsp90 and its client, as it is the "clamping" action that is predicted to provide Hsp90 with its molecular chaperoning activity. Conversely, the transition metal oxyanion molybdate binds to the same nucleotide binding site and locks Hsp90 in an ATP-bound conformation and stabilizes the Hsp90-client heterocomplex (*47, 48*).

Interestingly, the biologically essential ATPase activity of Hsp90 has been found to be quite weak with additional downstream residues providing both activity and inhibition (*49-52*). This suggests that Hsp90 ATPase activity is tightly regulated and most likely dependent on both client and cohort interactions. Additionally, the NAD is the binding site of Cdc37, an Hsp90 cohort required for client protein kinase interaction (*53*).

The LD of Hsp90 is the least conserved domain in both composition and length with it being completely absent in some Hsp90 homologs, such as HtpG and Trap1.

Co-chaperones:

- Cdc37
- p23, and relatives SGT1, RAR1, SIP, and Chp1
- Hop
- Hsp70 and Hsc70
- Human DnaJ homolog Hsj1
- S100A1
- Sse1
- Immunophilins: FKBP51, FKBP52, and Cyclophilin 40
- Other TPR proteins: PP5, TTC4, CHIP, UNC-45, CRN, WISp39, Tah1, NASP
- p97 vasolin containing protein (VCP)
- Aha1, possibly

Transcription Factors:

- Vertebrate steroid hormone receptors: GR, MR, ER, PR, AR
- CAR
- Cytoplasmic v-ErbA
- PPARα
- PXR
- Hap1
- HSF-1
- Mal63
- p53
- PAS family members: Dioxin receptor, Sim, HIF-1, 2, 3

Protein kinases:

- $pp60^{v\text{-}src}$, c-Src
- Src family tyrosine kinases: Lck, Yes, Fps, Fes, Fgr, Fyn
- Cyclin dependent kinases: Cdk4, Cdk6 Cdk9, Cdk11
- Raf-1, B-Raf, Ste11
- eIF-2α kinases: HRI, PKR, Gcn2, Perk
- ErbB2
- Akt/PKB
- Bcr-Abl
- Casein kinase II
- Chk1
- Death domain kinase RIP
- eEF-2 kinase
- Flt3
- GRK2
- IKK α,β,γ,ε
- Insulin receptor
- Integrin-linked kinase
- IRAK-1
- Ire1
- c-Kit mutant
- Lkb1
- MEKK1 and MEKK3
- MEK
- Mik1
- MLK3
- MOK, MAK, MRK
- c-Mos
- NIK
- PDK1
- Pim-1
- Pik1
- Plk1
- NAL kinase
- Slt2
- SSTK
- TAK1
- TBK1
- trkB
- VEGFR2
- Wee1, Swe1

Others:

- Apaf-1
- DNA polymerase α
- Mdm2
- Na^+-K^+-Cl^- cotransporter 1
- Rab-αGDI
- Hepatitis B RTase
- Telomerase

Table 1. **A represenatative list of current Hsp90 interacting proteins.** Proteins that assist Hsp90 in folding other proteins are referred to as co-chaperones. Whereas proteins that are folded by Hsp90 and its co-chaperones are referred to as client these include: transcription factors, protein kinases, and a variety of other proteins.

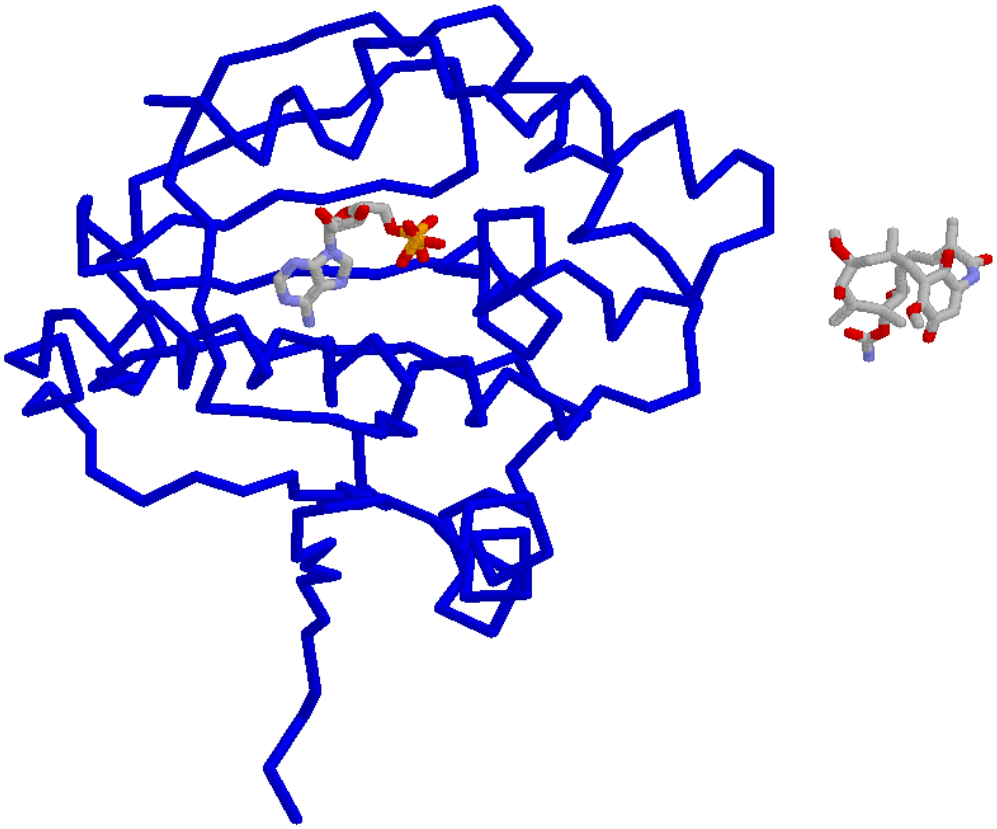


Fig. 1. **Structure of the Hsp90 N-terminal ATPase domain and geldanamycin.** X-ray crystal structures of (**A**) yeast NAD in the ATP bound conformation (PDB: 1AM1) (*39*), (**B**) structure of GA.

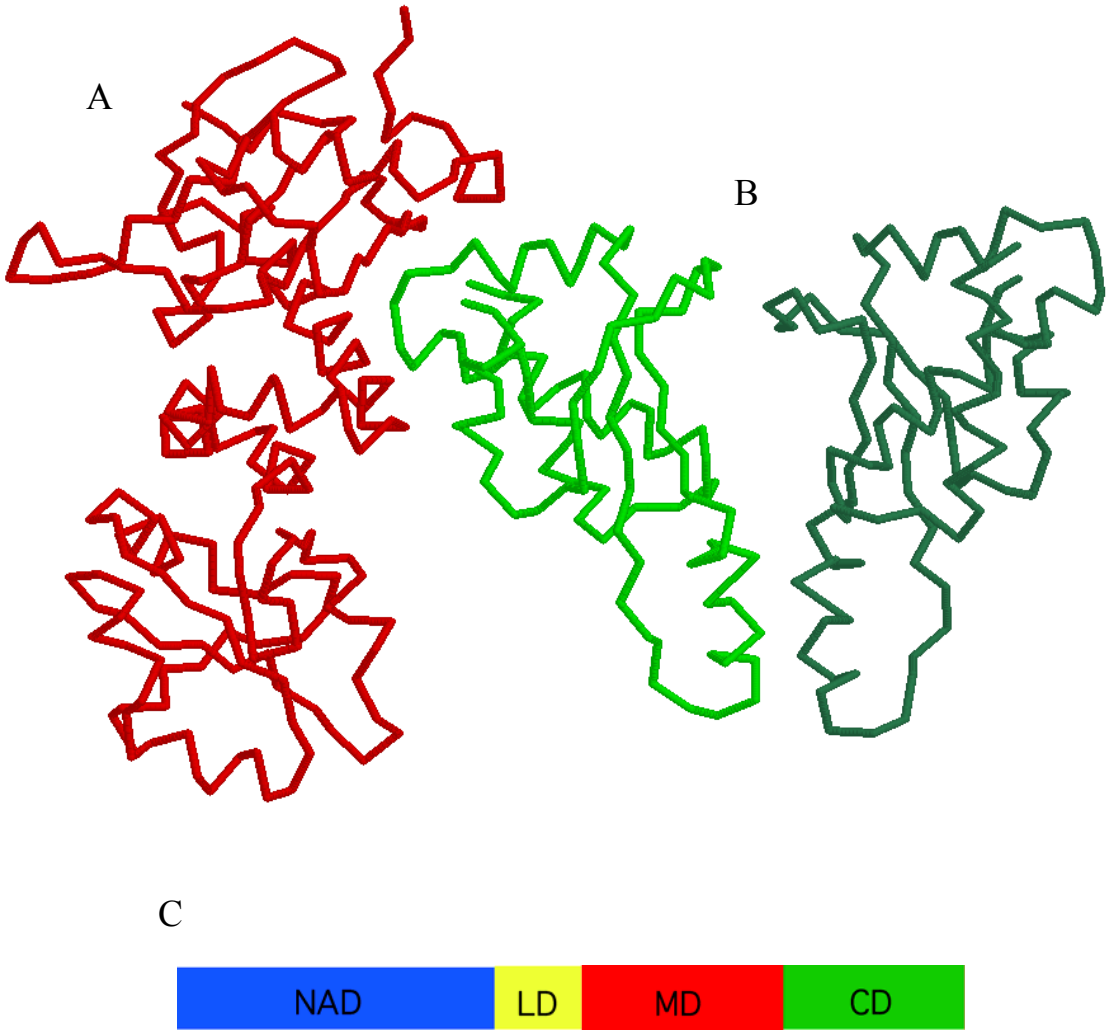


Fig. 2. **Structure of the Hsp90 middle domain and C-terminal domain.** X-ray crystal structures of (**A**) yeast MD (PDB: 1HK7) (*54*), (**B**) *E.coli* HtpG CD homodimerized (PDB: 1SF8) (*55*), (**C**) color representation of each Hsp90 domain.

Made up of primarily charged residues, the linker domain has been shown to contain two casein kinase II (CKII) phosphorylation sites and a possible nuclear localization sequence (NLS) (*56-58*). Furthermore, its presence in N-terminally truncated Hsp90 mutants, masks Hsp90's CD nucleotide binding site. However, despite these signs that it may play a role in regulating Hsp90 activity, the charged LD has also been shown not to be required for viability in yeast (*59*).

The X-ray crystal structure of the MD of yeast Hsp90 reaffirms the association of Hsp90 with the GHKL family of ATPases (*54*). Made up of a three layer α-β-α sandwich architecture the MD is predicted to contain the catalytic lysine that confers Hsp90's ATPase activity much like its GHKL relatives. The MD may also be directly involved in client/substrate interaction, while it is also the binding site of the peculiar cohort AhaI (*60*).

The 12 kDa CD is responsible for the majority of the interactions between Hsp90 monomers that drives Hsp90's dimerization. Dimer formation may also play an important role in regulating Hsp90's ATPase activity due to the fact that C-terminal truncation mutants of yeast Hsp90, which are unable to properly form dimers, exhibit greatly reduced ATPase activity (*52*). The C-terminal domain is also the binding site of numerous cohorts known as TPR (tetratricopeptide-repeat) proteins, which bind to the conserved C-terminal EEVD motif of Hsp90. An EEVD motif is also found at the C-terminus of Hsp70, due to the need of some TPR proteins to bind to both chaperones (*61-64*). Additionally, the CD of Hsp90 has been shown to contain a second nucleotide binding site, which plays a role in coordinating specific interactions of Hsp90 with its client proteins and modulates the nucleotide-binding affinity of its NAD (*65-68*).

However, whether this site functions as a second ATPase that helps drive protein folding or a site for allosteric regulation is not fully understood. Moreover, this is the binding site of the Hsp90 inhibitor novobiocin, which has also been shown to cause client protein dissociation (*68*).

Hsp70 and Co-chaperones

In order for Hsp90 to fully function as the "signal transduction chaperone" *in vivo*, the assistance of numerous partner proteins is required. These partner proteins known as cohorts or co-chaperones often possess molecular chaperone capabilities of their own, as well as inducible expression in response to many forms of cellular stress, such as heat shock. The roles of these co-chaperones in aiding Hsp90 have been shown to be quite diverse while some their roles are not completely understood. Some co-chaperones appear to help pre-fold the client protein and target them to Hsp90 for further folding, while also possibly modulating the ATPase and concomitant client folding activity of Hsp90. Whereas, it seems other co-chaperones interact directly with Hsp90, in order to direct client protein trafficking or remove certain post-translational modifications.

Hsp70 Chaperone Network

The highly conserved Hsp70 family of molecular chaperones makes up the centerpiece of a ubiquitous chaperone network that is able to fold a wide range of client proteins with the assistance of various co-chaperones. Several homologs of Hsp70 are found in prokaryotes, with the predominant homolog being known as DnaK. In

eukaryotes, Hsp70 homologs are present in the cytosol, ER, lysosome, mitochondria, and chloroplast. In mammals, two cytosolic isoforms are commonly expressed, the 72 kDa stress-induced Hsp70 and the 73 kDa constitutively expressed Hsc70 (*14, 15, 69, 70*).

Hsp70 is made up of three domains: an N-terminal 44 kDa ATPase domain, a 12 kDa peptide binding domain, and a 10 kDa C-terminal tail domain that often terminates with an EEVD motif like Hsp90. However, unlike Hsp90 in regards to substrate binding, ATP-bound Hsp70 is referred to be in the "fast on/fast off" conformation, while ADP-bound Hsp70 is referred to be in the "slow on/slow off" conformation. Also unlike Hsp90 whose client binding specificity is not well understood, Hsp70 has been determined to bind short extended linear polypeptides enriched in hydrophobic residues, most likely in order to prevent the aggregation of the exposed hydrophobic portion of a protein (*69-71*). Thus, Hsp70's binding specificity and activity concurs with Hsp70 being a general molecular chaperone aiding in the folding of an extremely wide variety of proteins.

To help fold this wide variety of proteins, Hsp70 is aided by several co-chaperones that regulate its ATPase activity. Activation of Hsp70 ATPase activity is stimulated by J-domain containing proteins, such as Hsp40s, which are homologous to DnaJ proteins in prokaryotes. As it is, Hsp40/DnaJ proteins possess their own chaperone activity and seem to bind to the substrate first and subsequently recruit it to Hsp70. The Hsp40 family consists of 44 members in humans, which allows DnaJ homologues to bind unique sets of substrates at different locations within the cell. This then provides a certain level of substrate specificity to Hsp70 which otherwise interacts with proteins rather non-specifically (reviewed in (*70, 72*).

Exchange of ADP and release of substrate is next facilitated by the nucleotide exchange factors, Bag and HspBP1, or their functional analog GrpE in prokaryotes (*73-75*). Additionally, in mammalian cells Bag-proteins and HspBP1 compete with the Hsp70 interacting protein, Hip, which stabilizes the ADP-bound form of Hsp70 and thereby prolongs substrate binding (*76*).

Six human Bag proteins have been reported so far. Bag-family proteins bind to the ATPase domain of Hsc70/Hsp70 via their C-terminal Bag-domain. Bag domains consist of approximately 110 amino acids that form three anti-parallel α-helices and are responsible for their nucleotide exchange activity. Several N-terminal domains or motifs that enable BAG-family proteins to partner with other proteins have been identified. These domains, like the additional domain found in DnaJ homologs are thought to enable BAG-family proteins to facilitate the release or the recruitment of specific client proteins to Hsc70/Hsp70 (*70*).

Hop

Identified as a component of the Hsp90 steroid hormone receptor complexes, Hop has been determined to provide a link between the Hsp70 chaperone network and the Hsp90 chaperone machine (*77, 78*). Comprised of three TPR (tetratricopeptide or 34 residue repeat) domains Hop is able to bind the EEVD motifs of both Hsp70 and Hsp90: hence its name Hsp90/Hsp70 organizing protein (Hop). Residues upstream of the EEVD motifs provide the binding specificity between each TPR domain, with the first domain (TPR1) binding Hsp70 and the middle domain (TPR2a) binding Hsp90 (Fig. 3). The polypeptide ligand for the third domain (TPR2b) has not been determined (*14, 61, 63*).

Further evidence indicates that Hop does not only passively link Hsp70 and Hsp90, but may also regulate their ATPase activity, with the binding of Hop stimulating Hsp70's and inhibiting Hsp90's ATPase activity, respectively (*79-81*). This property of Hop, in turn possibly coordinates protein client exchange between the two chaperone machines. The complex containing Hsp70, Hop, Hsp90 and the client protein is often referred to as the "intermediate complex" because it lies at the junction between the two major folding cycles of Hsp70 and Hsp90, with the "early complex" consisting of Hsp70 and its specific co-chaperones and the "late complex" consisting of Hsp90 and its specific co-chaperones (*14, 15*).

p23

A small yet complex protein, p23 is another Hsp90 co-chaperone that was originally identified in steroid hormone receptor complexes (*82, 83*). A member of the Hsp90 "late complex", p23 binds directly to dimerized ATP-bound Hsp90 and aids in facilitating client protein folding. Highly conserved from yeast to humans, p23 is comprised of a compact 8 β-sheet sandwich (Fig.3) with an acidic tail that is often phosphorylated (*84*). As it is, the acidic tail is needed for p23's passive chaperoning activity of denatured proteins along with the cooperative chaperoning of Hsp90 client proteins (reviewed in(*85*).

Evolutionary tracing methods and mutagenesis experiments predict that the direct binding of p23 to Hsp90 is carried out by the β-sheet sandwich of p23 and the dimerized N-terminal ATPase domain of Hsp90 (*86*). This supports data indicating that p23 plays a

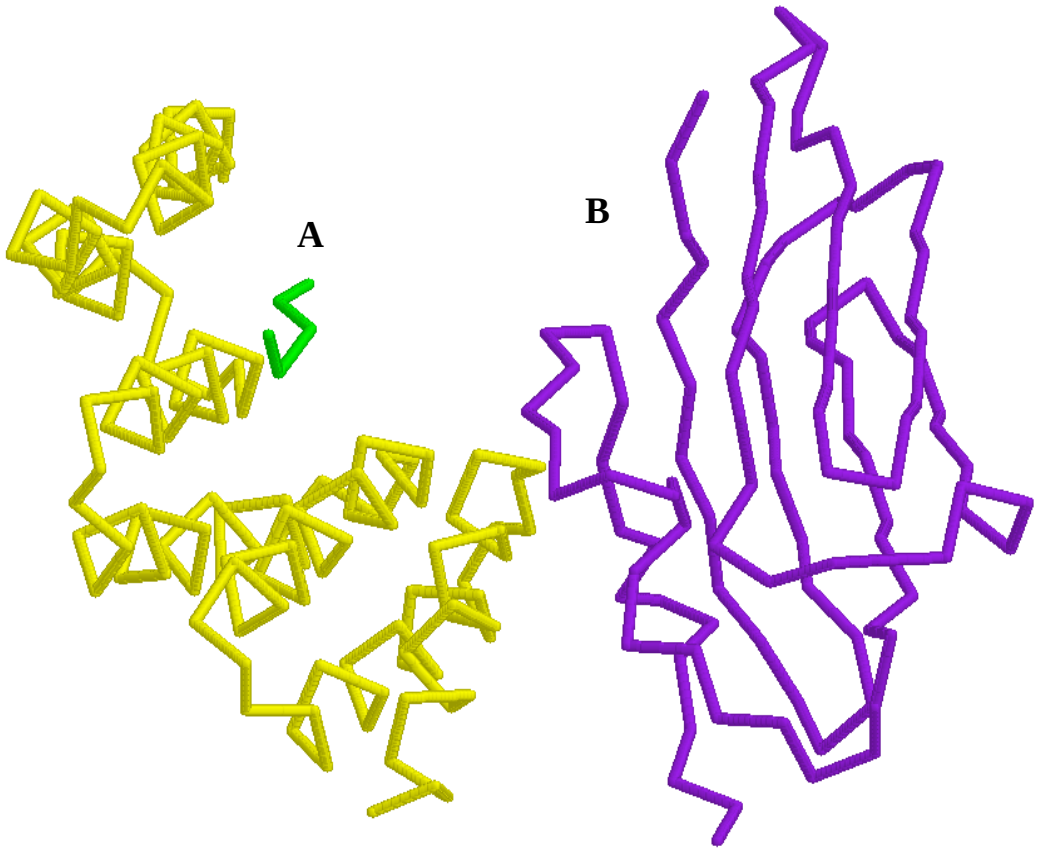


Fig. 3. **X-ray crystal structures of the TPR2a domain and p23.** (A) TPR2a domain of Hop (yellow)bound to the MEEVD motif of Hsp90 (green) (*64*); (B) N-terminal β-sheet domain of p23 (*84*).

critical role in stabilizing the interaction of ATP-bound Hsp90 with client proteins (*46*), although some reports suggest that p23 may also play a role in client protein release (*87*).

Other TPR Co-chaperones

Besides Hop, Hsp90 binds a large set of other TPR co-chaperones that are present only in the "late complex". These co-chaperones bind to the C-terminal EEVD motif of Hsp90 through their TPR domains, but contain other non-TPR domains that function in the folding or the maturation of the client protein. Immunophilins, which possess domains with peptidyl-prolyl-cis-trans isomerase (PPIase) activity upstream of their TPR domains, are required for proper steroid hormone receptor signaling and receptor transport. These immunophilins which are named for their ability to bind immunosuppressive drugs include FKBP51, FKBP52 (FK506-binding protein) and Cyp40 (cyclosporin A-binding protein) (reviewed in (*15, 88, 89*). PP5 (protein phosphatase 5) is a TPR protein whose phosphatase activity has been found to modulate the maturation client protein kinases (*90*). Another "late" TPR protein, TTC4 is predicted to help assemble DNA replication complexes and is currently being studied in our lab.

AhaI

The AhaI/HchI proteins are the most recent additions to the Hsp90 chaperone machine. HchI, an abbreviated homolog of AhaI, was originally identified as a high copy suppressor of the E381K mutant Hsp90 in yeast (*91*). Moreover, AhaI has been shown to stimulate the ATPase activity of yeast Hsp90 by up to 12 fold (*92, 93*). Further studies have also determined that AhaI directly binds to the middle domain of Hsp90 with the

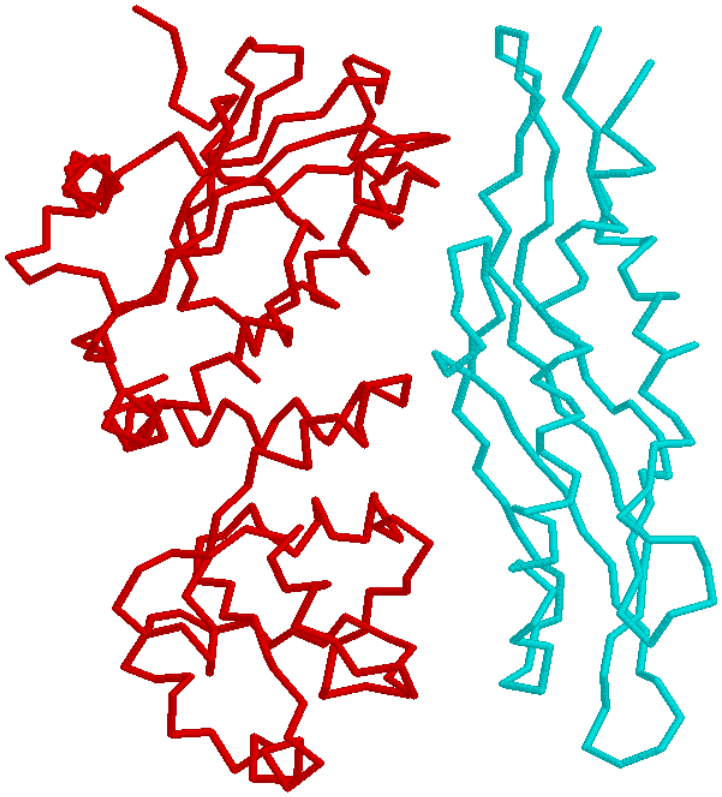


Fig. 4. X**-ray crystal structure of AhaI bound to the Hsp90 MD.** AhaI (cyan) binds to the "backside" of the MD (red) of Hsp90. In the manuscript describing the interaction another orientation was shown in which AhaI bound to the "front side" of the MD (*60*). However, this orientation can not be found in the PDB.

crystal structure showing two different orientations (Fig. 4) (*54, 60*). Additionally, published reports suggest that AhaI helps Hsp90 facilitate both steroid hormone receptor and protein kinase maturation (*91, 94, 95*). These findings however conflict with the current work done in our lab which suggests that AhaI interacts with Hsp90 in a manner similar to a client protein, and has no direct interaction with Hsp90-kinase or Hsp90-steroid hormone receptor complexes.

Cdc37

Cdc37 or p50 is currently understood to be the co-chaperone that is specifically responsible for the partial folding and recruitment of client protein kinases to the Hsp90 chaperone machine (reviewed in (*96, 97*)). Yet, how Cdc37 recognizes client kinases and specifically modulates the chaperoning process with Hsp90 is not understood. Initially identified as a cell-division cycle mutant that arrests *S. cerevisiae* in G1 (*98*), Cdc37 was later found in mammalian cells to physically interact with Hsp90 and the Rous sarcoma viral kinase $pp60^{v\text{-}src}$ (*99*). Cdc37 since then has been found to interact with a variety of other protein kinases in concert with Hsp90, while also being shown to be essential for the viability of *S. cerevisiae* (*100*), *Caenorhabditis elegans* (*101*) and *Drosophila melanogaster* (*102*).

The importance of Cdc37 for viability is further illustrated by its requirement to support the function of several client protein kinases that transduce pro-growth signals. Determined to be the only co-chaperone whose level of expression can considerably affect cell proliferation (*103*), Cdc37 expression has been found to be drastically higher in human prostatic cancers and pre-malignant neoplasias (*104*). Ectopic overexpression

in transgenic mice has been shown to promote the formation of mammary tumors with Cdc37 also working in synergy with c-Myc in the transformation of salivary and testicular tissue (*105*). These studies in turn have implicated Cdc37 as an indirect yet highly influential proto-oncogene that helps maintain or possibly promote many of the pro-growth signals upon which tumors become dependent on.

Continuously required to maintain the population of client kinases, Hsp90 and Cdc37 can specifically interact with both nascent kinases emerging as polypeptides from the ribosome and fully mature protein kinases that depend on Hsp90 and Cdc37 for their ongoing cellular stability (*106*). These interactions, however, do not simply reflect Cdc37 acting as a scaffold, linking the client kinase to Hsp90. Experimental data indicate that Cdc37 also modulates Hsp90 nucleotide-mediated conformational switching which causes both chaperones to associate with the client kinase in a synergistic manner. Thus, the interaction between Cdc37 and Hsp90 and the client kinase is stabilized by the presence of both chaperones. In addition, the interaction between Cdc37 and Hsp90 themselves is stabilized by the presence of the client kinase in the complex (*107*). These complexes have proven to be quite stable, being able to withstand washing with high ionic strength buffer. The ability of kinases to form these salt stable chaperone complexes is dependent on the ATP-induced dimerization of Hsp90's NAD into its "molecular clamp" conformation, which somehow secures both Cdc37 and the client kinase in a hydrophobic embrace (*107, 108*). This complex is then disrupted by the hydrolysis of the ATP to ADP causing the release of both the client kinase and Cdc37. Similarly, the high affinity binding of GA to Hsp90 causes the same constitutive effect as

ADP binding (*107*), which often leads to client kinase destabilization within the cell (reviewed in (*23*)).

Interestingly, Cdc37 itself has also been found to inhibit the ATPase activity of Hsp90 much like Hop (*109*). Although, exactly how this inhibition helps modulate Hsp90's nucleotide-mediated conformational switching, and therefore the chaperoning process is not completely understood. This activity of Cdc37 suggests that it would allow for a prolonged interaction with the client kinase and Hsp90, therefore allowing the client kinase to eventually fold into its proper conformation. Additionally, Cdc37 is predicted to work in conjunction with other "late co-chaperones" such as the immunophilins, PP5, TTC4, and p23 (*90, 96, 97, 107*). Furthermore, although it has been reported in the literature that Cdc37 is able to bind Hsp90 along with AhaI in vitro (*95*), current work in our lab has shown that Cdc37 is not present in AhaI-Hsp90 complexes.

Despite it being essential for viability, Cdc37 is poorly conserved throughout evolution sharing only 20.5% amino acid identity between the mammalian and yeast homologues. The lack of conservation is also denoted by the differences in size with yeast being 506 residues and human only 378 residues. The most conserved region of Cdc37 is the first 40 amino acids which is over 50% identical between yeast and human. Notably, this N-terminal region has been determined to be the kinase binding domain (KBD) (*106, 110*). Truncated mutants of Cdc37 containing the KBD are able to affect client kinase folding and stability in a dominant-negative manner by binding to the kinase yet not targeting it to Hsp90 for chaperoning (*106, 111, 112*). Also, although Cdc37 homologues have been found in every metazoan studied no plant homologues have been currently identified. However, our lab has identified an interesting *Arabidopsis thaliana*

protein that contains an N-terminal TPR domain followed by a region that resembles the first 40 amino acids of yeast and human Cdc37. Studies to resolve whether this protein truly is a plant Cdc37 homologue are currently being conducted.

Domain dissection work has determined that human Cdc37 consists of three defined regions: the previously mentioned kinase binding domain (KBD) [residues 1-126], the Hsp90 binding domain (HBD) [residues 127-282], and a third domain (D3) [residues 282-378] whose function is unknown. Deletion of the first eight amino acids of the KBD has shown that the very N-terminus of Cdc37 is critical for kinase binding activity. Alanine scanning mutagenesis of these first eight residues indicates that both Tyr4 and Trp8 are needed for kinase binding with Trp8 also playing a role in Hsp90 interaction (*108*). The KBD moreover, contains a highly conserved CKII phosphorylation site at Ser13. Phosphorylation of this site has proven to be functionally significant in allowing Cdc37 to interact with client kinases and modulate Hsp90's nucleotide-mediated conformational switching which allows for the formation of the salt stable client-chaperone complex (*108*). Interestingly, CKII itself is functionally dependent on Cdc37, and thus creates a positive feedback loop between the client kinase and the co-chaperone (*113, 114*).

The crystal structure of the human HBD of Cdc37 with the yeast NAD of Hsp90 has been solved and depicts Cdc37 binding to the mobile "lid" segment of the ATP binding cleft of Hsp90 (Fig. 5) (*53*). This along with Cdc37 directly inserting Arg167 into the ATP binding cleft suggests how Cdc37 is able to inhibit the ATPase activity of Hsp90. The crystal structure also depicts the Cdc37 HBD as a dimer that binds Hsp90 on either face, which would further prevent the N-terminal domains of Hsp90 from forming

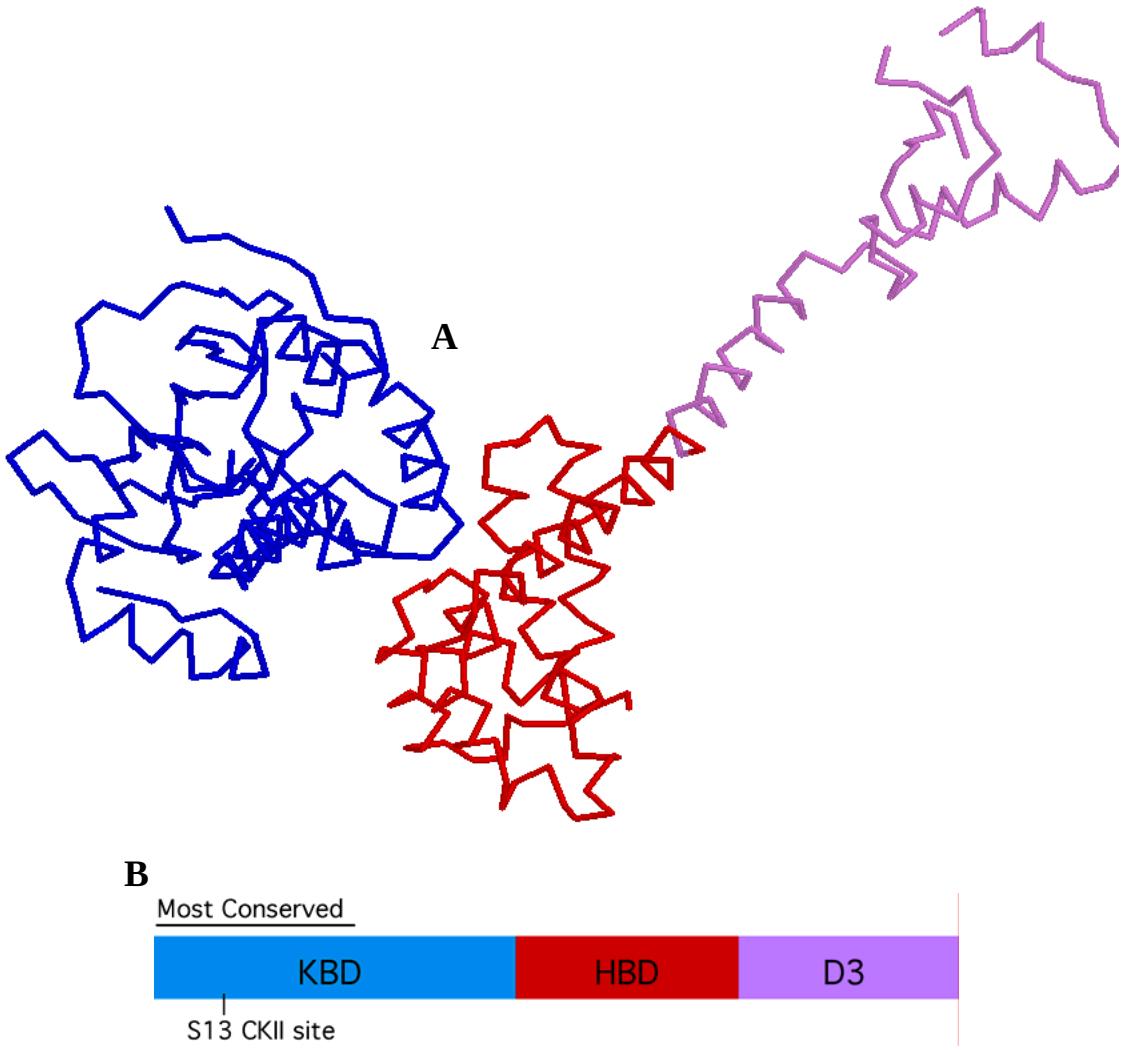


Fig. 5. **Structure of Cdc37.** (**A**) X-ray crystal structure of human Cdc37 HBD and D3 bound to yeast Hsp90 NAD (*53*). Cdc37's KBD is not shown. (**B**) Color representation of Cdc37's functional domains.

their own dimer upon ATP binding. This conformation is proposed to hold open the "jaws" of the "molecular clamp", and is predicted to facilitate the loading of the client kinase. D3, which was also partially solved in this crystal structure, is a globular helical bundle following the extended helix of the HBD. What the function of this domain is remains unknown but it is speculated that the large extended helix may indeed be a crystallization artifact. In yeast both the HBD and D3 are expendable, in part. Cdc37 mutants truncated after the HBD function the same as wild-type, whereas mutants truncated after the KBD are only partially functional. The true relevance of this crystal structure to mammalian Cdc37, however should be considered with caution since these domains are the least conserved throughout evolution.

The HBD of Cdc37has been discovered in another protein called Harc (Hsp90-asociating relative of Cdc37). A 35 kDa protein, Harc is 31% identical to human Cdc37 with the HBD's being 62% identical (*115*). The other two domains of Harc hold little homology to those of Cdc37. Originally identified in humans, Harc has been found to exist only in vertebrates with its mRNA expressed primarily in the liver, skeletal muscle, kidney and heart (*115*). Curiously, little is known about Harc's function and whether or not it acts similarly to Cdc37 and recruits a specific population of client proteins to be chaperoned. Currently, our lab is studying Harc in hopes of answering this question and to further determine its function as an Hsp90 co-chaperone.

The mystery of Cdc37 persists in its interactions with its clients, as it is not understood why some kinases are clients of Hsp90 and Cdc37 and some kinases are not. Equally interesting is the fact that Cdc37 associates with non-kinase Hsp90 clients. For example, Cdc37 interacts with the ligand binding domain of the androgen receptor in a

manner reminiscent of its client kinase interactions, but not with the closely related glucocorticoid receptor (GR) (*112*). Cdc37 also has been found to specifically interact with the reverse transcriptase of the duck hepatitis B virus, which is predicted to be structurally similar to the client kinase Raf-1 (*116*).

Hsp70/Hsp90 Chaperone Cycle

The Hsp70/Hsp90 chaperone cycle was originally described by Smith et al. to explain the mechanisms and interactions of steroid hormone binding activity for both the glucocorticoid and progesterone receptors (*117*). In rabbit reticulocyte lysate, assembly of receptor-Hsp90 heterocomplexes was found to require ATP, Mg^{2+}, a monovalent cation such as K^{+}, and Hsp70. Further work identified Hsp40, Hop, and p23 as components that each associated with the chaperone heterocomplex at different points of the cycle. Eventually, it was then shown that it was possible to assemble a steroid hormone receptor-Hsp90 heterocomplex *in vitro* using purified proteins. This minimal system sometimes referred to as the *foldosome* consists of Hsp40, Hsp70, Hsp90, Hop, and p23. However, although this five purified protein system has been essential in elucidating the Hsp70/Hsp90 chaperone cycle it has been used primarily to describe the chaperone interactions with steroid hormone receptors, with much still to be determined about other client proteins, such as kinases.

Client proteins folded by the Hsp70/Hsp90 chaperone cycle are processed and passed from one complex to another with different sets of chaperones in each complex (Fig. 6). In the "early complex" Hsp70 and its cohorts bind to the hydrophobic motifs of the client polypeptide allowing the core of the polypeptide to collapse. The partially

folded client is then passed on to Hsp90 and its cohorts within the "intermediate complex". The Hsp90 chaperone machine then folds and rearranges the tertiary and/or quaternary structure of the client protein in the "late complex". The folded client protein is then released to carry out its function. However, some client proteins are inherently unstable and are taken back into the cycle repeatedly until they are properly modified or bind ligand, as is the case for steroid hormone receptors.

The Hsp90/Hsp70 chaperone process is first initiated by the binding of an Hsp40 or a DnaJ homologue to the client polypeptide, which may exist as either a nascent polypeptide or a mis-folded protein. The client polypeptide is then recruited to Hsp70 where Hsp40 stimulates the ATPase activity of Hsp70 with its J-domain causing Hsp70 to bind tightly to the hydrophobic stretches of the polypeptide. This is referred to as the "early complex". At this point several different things can then happen depending on which co-chaperone binds next. If the nucleotide exchange factor Bag-1 binds it causes Hsp70 to exchange bound ADP for ATP, and the client protein to be released from the "early complex". However, if the TPR domain co-chaperone Hip binds to Hsp70 instead, the "early complex" will be stabilized in the ADP bound form. It is likely that Hsp40 in these complexes is displaced since all three co-chaperones associate with the N-terminal ATPase domain of Hsp70, although Hsp40 in some instances may still associate with the polypeptide through its client binding domain.

If the TPR protein Hop binds, the "early complex" becomes the "intermediate complex", as Hop bridges Hsp70 to Hsp90. In the "intermediate complex" it may be possible for Hsp40 to be present since Hop coordinates both Hsp70 and Hsp90 by binding to their C-terminus EEVD motifs. Additionally, the observed

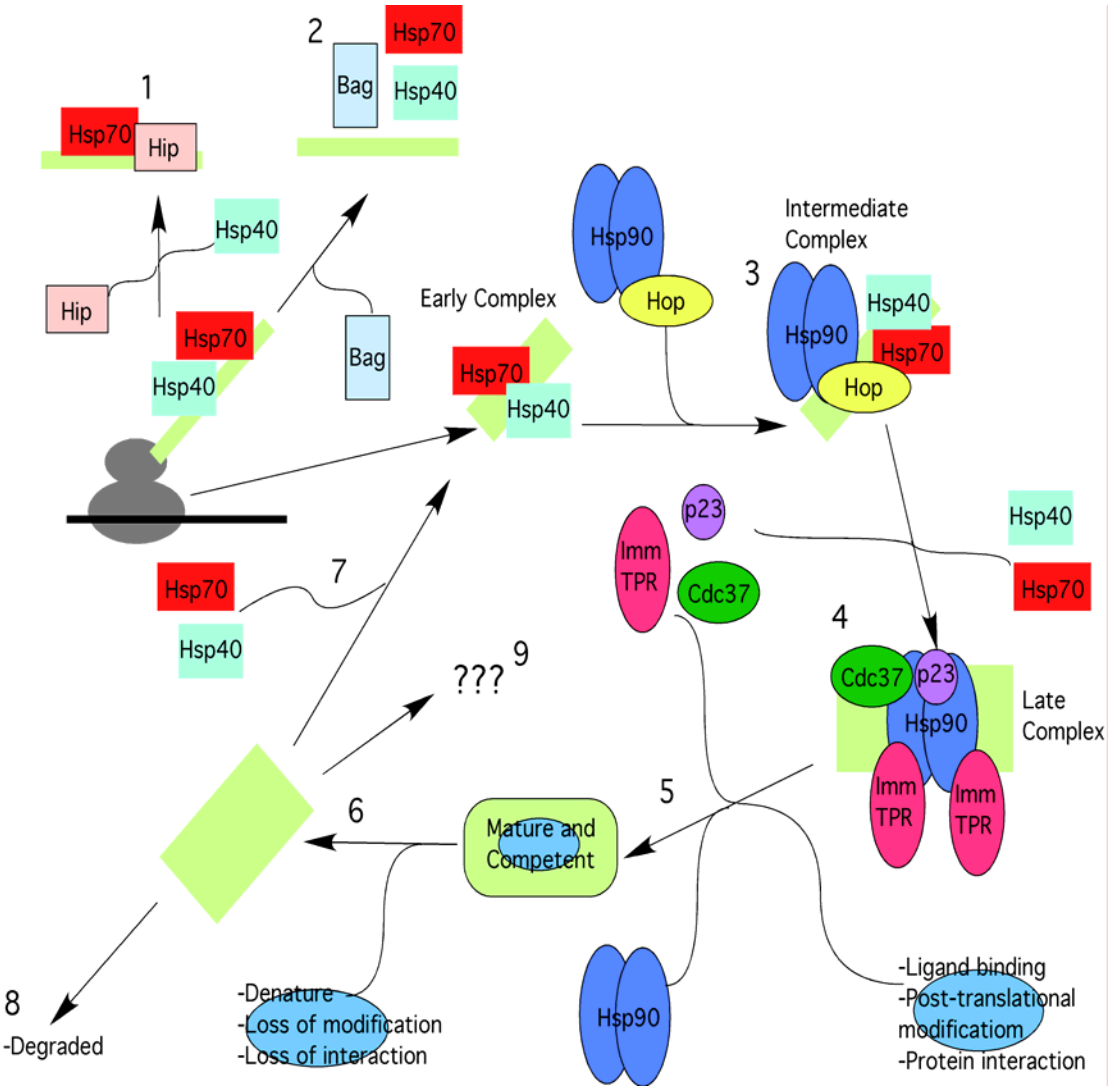


Fig. 6. **Hsp70/Hsp90 Chaperone Cycle description adapted from (15). "**Early complex" chaperones such as Hsp40 and Hsp70 associate with nascent polypeptides as they emerge from the synthesizing ribosome. **(1)** Hip displaces Hsp40 and maintains Hsp70 in the ADP bound state, tightly binding the polypeptide, or **(2)** Bag displaces Hsp40 and causes Hsp70 to release both the polypeptide and ADP, or **(3)** Hop binds Hsp70 via its TPR1 domain and recruits Hsp90 to form the "intermediate complex". **(4)** Hsp90 dimerizes its NADs upon ATP binding while "late complex" chaperones such as p23, Cdc37, PP5, and immunophilins associate. Hsp70 and Hsp40 are then allowed to dissociate. **(5)** The client protein is further folded by Hsp90 and its cohorts into a mature protein. In this complex the client protein may also be acted upon by other components such as kinases and phosphatases or bind ligand upon release. **(6)** Although, the mature client may be destabilized by inherent instability or loss of ligand and/or post-translational modification. **(7)** Hsp40 and Hsp70 recognize the unstructured polypeptide and recruit it back into the early complex, or **(8)** the unstable protein is ubiquitinated and degraded by the proteosome or some other form of proteolysis, or **(9)** the protein is recruited back into the cycle by some other means.

stoichiometry of this Hsp90: Hop: Hsp70 complex is 2:1:1, suggesting that only one of the Hsp90 dimer is interacting with the Hop-Hsp70-client heterocomplex.

The transition from the "intermediate complex" to the "late complex" is not well defined, but consists of events that allow the partially folded client protein to be transferred from Hsp70 to Hsp90. The coordinated release of the client protein by Hsp70 is followed by the dissociation of all the "early complex" components (Hsp70, Hsp40, Hip) and the "intermediate complex" component Hop. On the other hand, the coordinated binding of the client protein by Hsp90 is followed by the association of several "late complex" components such as p23, immunophilins, PP5, and Cdc37.

When GA or any of its analogs are present, Hsp90 is found to be in the ADP-bound conformation and therefore unable to form productive "folding" complexes with the client protein. This is demonstrated by the inability of Hsp90 to form "late complexes" with the client protein that are stable in the presence of high ionic strength. Moreover, since the "intermediate complex" is the first opportunity for Hsp90 to interact tightly with the partially folded client protein, GA inhibition is predicted to block the Hsp70/Hsp90 chaperone cycle at this point. This, in turn, causes the observed increase in Hsp70 interaction with the clients, due either to its inability to hand-off partially folded client proteins to Hsp90, or due to the increased population of mis-folded proteins in the cytosol. In the presence of GA, unstable client proteins are then subject to higher rates of proteolytic degradation, typically carried out through the ubiquitination/ proteasome pathway (*20, 118*).

"Late complexes" are characterized by the stable binding of Hsp90 and its co-chaperones to the client proteins. In the "late complex", Hsp90 binds ATP and dimerizes

its NADs into the "clamp" conformation. The formation of "late-complexes" also involves the recruitment of p23 to Hsp90. Once p23 is bound to Hsp90, Hop binding is diminished. As p23 tightly binds to the dimerized NADs of Hsp90, the client-chaperone heterocomplex becomes stabilized. This is concurrent with or followed by, the binding of the TPR co-chaperones to the now vacated EEVD motifs of Hsp90. These co-chaperones are predicted to carry out their specific tasks while the client protein is held by Hsp90. For steroid hormone receptors, this "late complex" conformation is required for the opening of the ligand binding cleft and subsequently the ligand binding activity of the receptor.

Cdc37 is also present in "late-complexes" formed between Hsp90 and its clients. However, since it is required for the recruitment of clients, especially protein kinases, to the Hsp90 chaperone machine, Cdc37 may also be present in earlier complexes and possibly play a role similar to Hop in mediating the client exchange from Hsp70 to Hsp90. Finally, once the Hsp90 chaperone machine has been fully assembled, the client protein is folded and released as a "mature" protein. When ATP hydrolysis occurs and how the "late complex" chaperone machine is disassembled remains to be characterized.

The release of the client protein and the dissociation of the "late" complex chaperones, however, can be prevented by the addition of molybdate. Like GA, molybdate inhibits Hsp90 function, but instead of locking Hsp90 in the ADP bound "fast on/fast off" conformation molybdate appears to lock Hsp90 in its "slow on/slow off" conformation. This causes the Hsp90 chaperone machine to bind the client protein in a high-affinity salt-resistant manner: a characteristic that is useful in studying the interactions between the Hsp90 chaperone machine and the client protein (*48, 68, 106,*

108, 119). However, unlike steroid hormone receptors, which require molybdate to stabilize their interaction with the Hsp90 chaperone machine (*117*), full length protein kinases can form highly stable salt-resistant heterocomplexes with the "late" Hsp90 chaperone machine independent of molybdate (*48, 108*). Additionally, because molybdate is able to lock Hsp90 in its "slow on/slow off" conformation (*48*), it has been speculated that molybdate mimics the gamma phosphate of the hydrolyzed ATP bound to Hsp90. This has led to the hypothesis that the release of the hydrolyzed gamma phosphate from the Hsp90 nucleotide binding cleft is an important step in Hsp90's reaction cycle, and that phosphate release, not merely ATP hydrolysis, may be the trigger that stimulates client protein dissociation.

Upon release, the folded client protein is then capable of functioning properly, but by design it may eventually be reloaded into the Hsp70/Hsp90 chaperone cycle if it becomes unstable due to either a loss of ligand binding, loss of a post-translational modification or an inability to bind the correct partner protein. This appears to be a common characteristic that provides the Hsp70/Hsp90 chaperone cycle with a certain degree of regulation over most all signal transduction pathways.

Protein Kinases

In eukaryotic cells, protein kinases play a major role in modulating every biological function that allows the organism to adapt and survive. Cell division, metabolism, growth, differentiation, and apoptosis are all regulated by signal transduction pathways that include one or more protein kinases. Protein kinases use ATP to covalently attach a phosphate group to a serine, threonine, or tyrosine on the substrate

protein, thereby altering the protein's conformation and electrostatic topology. These alterations can then affect the substrate protein's function, activity, subcellular localization, and/ or cellular stability. Moreover, these effects can also alter the interactions of the phosphorylated protein with other proteins, by altering the activity of the substrate protein itself and/or its partner proteins. Furthermore, since each signal transduction pathway must dynamically adjust to the ever changing cellular environment, the ability to remove the phosphate group from the substrate protein is essential: a reaction which is carried out by a family of enzymes known as phosphatases. Thus, the specific phosphorylation state of a cell's proteome and its ability to maintain homeostasis is balanced by the activities of both protein kinases and phosphatases.

In a cell, the goal of most signal transduction pathways is ultimately to alter gene expression. This is, in order, to either adapt to the environment or to change the properties of the cell itself so that it can better serve the whole organism. Organized as a network of detectors, transducers, and effectors each signal transduction pathway consists of several tiers with a different kinase at each tier. Kinases at the higher tiers are activated by receptors or are receptors themselves that transduce signals by activating kinases at the intermediate tiers. The activation of downstream kinases is typically achieved by phosphorylating the target kinase, in many cases within its activation loop. These intermediate tier kinases then transduce this signal by phosphorylating and thereby activating lower tier kinases. Finally, the lower tier kinases go on to phosphorylate and activate the effector proteins.

The transduction of the signal along these pathways occurs in a cascade like manner with each kinase capable of phosphorylating numerous downstream substrates.

This mechanism, in turn, allows the original signal to be amplified exponentially. Each kinase may also phosphorylate several different species of substrates, thereby broadening the effect of the original signal. When members of a different signaling pathway are activated it is referred to as crosstalk between the two pathways. These crosstalk events frequently serve to regulate the signal through a positive or negative feedback mechanism. In the end the signaling pathway is eventually reset as each activated kinase is de-phosphorylated and inactivated by a phosphatase.

Protein kinases make up at least 1-2% of all the proteins encoded by eukaryotic genomes, making them the third most common protein domain in the human genome (*120*). This type of prevalent expression combined with the reality that abnormal protein kinase activity often leads to disease has led to protein kinases emerging as major drug targets. As it is, one-third of all newly validated drug targets in the pharmaceutical industry are protein kinases (*121*).

For general purposes protein kinases can be classified into two groups based on substrate specificity: tyrosine kinases (PYK) and serine/threonine kinases (PSTK). PYKs are expressed predominantly in metazoans and are responsible for transducing the complex intercellular signals of higher eukaryotes. Also PYKs are significantly liable for many human diseases, as PYKs constitute over 80% of all known proto-oncogenes. PYKs are typically found at the outer membrane of the cell with 58 of the 90 PYKs encoded by the human genome being cell surface receptors with cytoplasmic kinase domains (*122*). These receptor PYKs function by binding extra-cellular ligands, which induce receptor oligomerization and subsequently the trans-autophosphorylation of their cytoplasmic domains. This then activates the receptor PYKs towards their intracellular

substrates or leads to the assembly of signal transduction modules. These activated substrates or signaling modules then go on to propagate the signaling pathway. The remaining non-receptor PYKs, such as the lymphoid cell kinase (Lck), also localize to the cytoplasmic side of the plasma membrane due to the presence of covalently bound fatty acids. These peripherally associated plasma membrane kinases interact with and are regulated by other cell surface receptors, where they also function in transducing receptor-mediated signals.

PSTKs, on the other hand, are found in both metazoan and unicellular eukaryotic organisms. PSTKs, such as Cdk2 and JNK, are responsible for the regulation and transduction of intracellular signals. Thus, PSTKs typically function downstream of PYKs in protein kinase cascades that make up the integrated signal transduction pathways.

Protein Kinase Structure

The overall fold of the protein kinase catalytic domain is remarkably conserved across all eukaryotic protein kinase families. Consisting of 250-300 amino acids, the kinase domain folds into a two-lobed structure that can be preceded and/or followed by any number of regulatory domains. The two-lobed structure can be further divided into 12 conserved structural motifs, as described by Hanks and Hunter (Fig. 7 and Table 2) (*123*). These motifs are defined as regions containing both the conserved amino acids and 2^0 structure that are essential for the function of the kinase. The smaller N-terminal lobe (NL) is composed of motifs I-V and is responsible for binding and orientating the ATP molecule, while the larger more stable C-terminal lobe (CL) is composed of motifs

V-XI and is involved in the binding of the peptide substrate and initiating phosphotransfer. Structural motif V links the NL and CL together with portions of it often included in either lobe.

Protein Kinases Studied

The three protein kinases that are primarily studied in this project are the lymphoid cell kinase (Lck), the cyclin-dependent kinase 2 (Cdk2) and the c-Jun N-terminal kinase 1 (JNK1). Currently, Hsp90 and Cdc37 have been shown to physically interact with Lck (*124*), but not JNK1 or Cdk2 (*125*). Genetic evidence has, however, strongly suggested that Cdk2 interacts with Hsp90 and Cdc37 (*126*). A brief description of the structure, function, and role in signal transduction of each kinase in provided.

Lck

Lck is a 56 kDa Src-family tyrosine kinase that is required for proper T-cell antigen receptor (TCR) signal transduction (reviewed in (*127*). Lck is made up of six defined domains: 1) an N-terminal fatty acid attachment domain, 2) a unique domain; 3) a Src-homology 3 (SH3) domain; 4) an SH2 domain; 5) the tyrosine kinase domain; and 6) a C-terminal inhibitory tail domain (CITD). The N-terminal fatty acid attachment domain is the site where Lck is myristoylated and palmitoylated, which allows Lck to localize to the plasma membrane and translocate to lipid rafts, respectively. The unique domain utilizes the coordination of a Zn^{2+} ion to mediate its interaction with the T-cell co-receptors, CD4 and CD8. The SH3 domain is a poly-proline binding domain that aids in the inhibition of Lck and the binding and localization of signal transduction particle

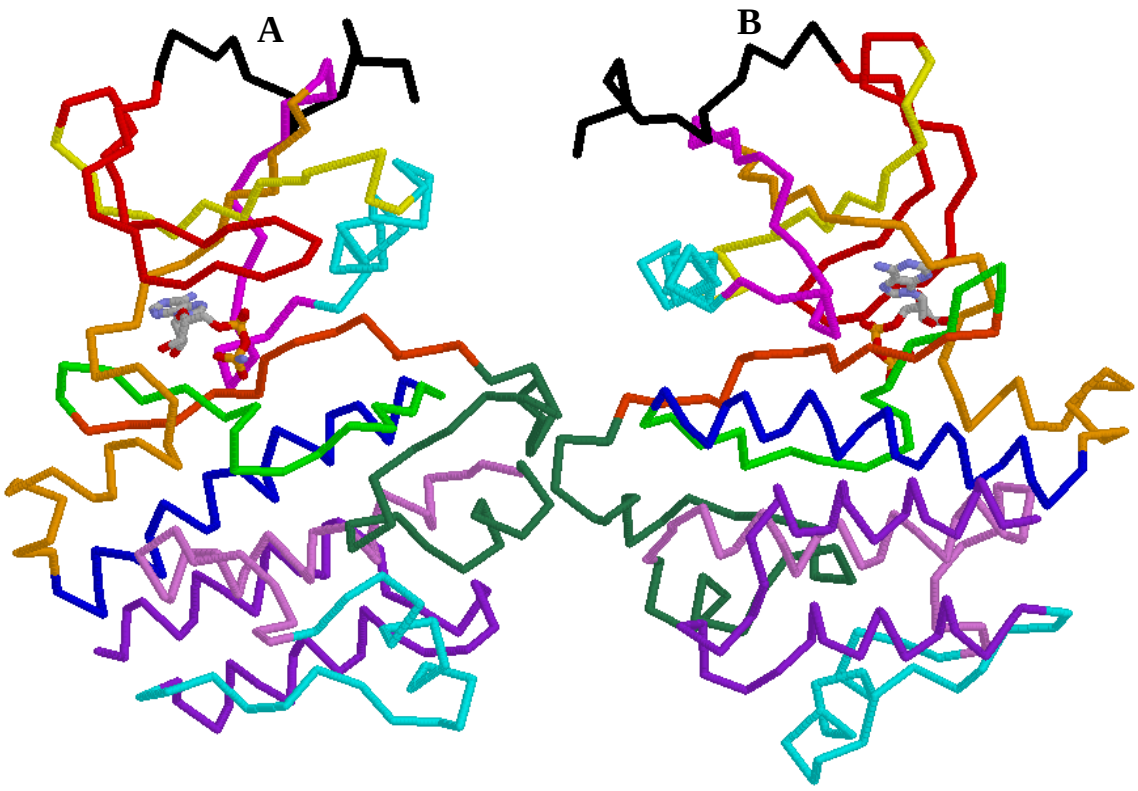


Fig. 7. **Structure of the catalytic kinase domain of Lck.** Using the kinase domain of Lck as a reference each structural motif is depicted by a different color and explained in Table 2. The kinase domain is shown with its ATP binding cleft facing either **(A)** toward or **(B)** away from the viewer.

Structural Motif	Residues	Color	Information
I	245-266	Red	Two β-strands which contain the G-box motif, GXGXXG. Covers and anchors the non-transferable phosphates of ATP.
II	267-280	Yellow	β-strand 3 which contains an invariant catalytic Lys.
III	281-295	Cyan	α-helix C which contains an invariant Glu needed for stabilizing the α and β phosphates of ATP.
IV	296-310	Magenta	Hydrophobic β-strand 4 with hinge-loop motif.
V	311-336	Orange	Connects the NL and CL, hydrophobic random coil and α-helix D, may also contain large regulatory insertion sequences.
VIA	337-358	Blue	Large α-helix E where hinge-loop motif pivots on.
VIB	359-375	Green	Catalytic loop (HRDLK/RXXN) and β-strand 6.
VII	376-388	Red orange	β-strand 7 contains the DFG motif, which coordinates Mg^{2+} or Mn^{2+} ions.
VIII	389-415	Green blue	Contains the APE-box motif which salt-bridges with an invariant Arg in XI. Plays a major role in substrate recognition and activation, may be phosphorylated by activating kinases.
IX	416-443	Violet	Large α-helix F contains an invariant Asp which stabilizes the catalytic loop.
X	444-464	Cyan	α-helix G poorly conserved, function is unclear.
XI	465-501	Purple	α-helices H and I contain an invariant Arg which salt-bridges with Glu in VIII.

Table 2. **Summary of the Structural Motifs of Protein Kinases.** Each structural motif is designated by a roman numeral according to Hanks and Hunter. A different color is given for each motif according to Fig. 7. A brief description of each motif is given with the β-sheets being numbered 1-7 and the α–helices designated A-I.

components containing poly-proline II type helical motifs. The SH2 domain is a phospho-tyrosine binding domain that binds to phosphorylated Y505 located in the CITD, which in turn inhibits Lck kinase activity. The SH2 is also responsible for docking of Lck to signal transduction particle components containing phosphotyrosine. The tyrosine kinase domain functions as previously described.

The activation of Lck and transduction of the TCR signal are highly dependent on two factors; the phosphorylation state of Lck and its localization (reviewed in (*127*). When Y505 of Lck is phosphorylated by Csk1 the SH2 domain intramolecularly binds to the CITD and locks down the tyrosine kinase domain. This interaction also causes the SH3 domain to bind to a poly-proline motif within Lck that further inhibits kinase activity. Once Y505 is de-phosphorylated by the phosphatase CD45, Lck is said to be in its "primed" state and is capable of being activated. Upon TCR activation Lck is recruited to the receptor complex via its interactions with the CD4 and CD8 co-receptors. Here Lck clusters together and is believed to trans-autophosphorylate Y394 in its activation loop. Now a fully activated kinase, Lck phosphorylates specific motifs on co-receptors CD3, TCRζ, and the kinase Zap70 that eventually culminate in T-cell activation.

Cdk2

Cdk2 is a 298 amino acid serine/ threonine kinase that is essential for proper cell cycle progression (reviewed in (*128*) . Structurally simple, much like most members of the cyclin-dependent kinase family, Cdk2 is made up of a kinase domain without any flanking regulatory subunits (*129*). Cdk2 kinase activity and substrate specificity

however are regulated by the coordinated association with the Cyclin E and Cyclin A proteins. Cyclins, which are systematically expressed and degraded throughout the cell cycle, bind to Cdk2 and stabilize it in an activatable conformation. Cdk2 can then be phosphorylated by CAK (cdk activating kinase, also known as Cdk7/Cyclin H) on T160 in its activation loop to become an active kinase. Subsequently, depending upon the cyclin with which it is associated, Cdk2 is able to phosphorylate its specific set of substrates. When bound to Cyclin E, Cdk2 substrates include Rb, p27, NPAT, and Cyclin E itself. Phosphorylation of these proteins promotes the initiation of DNA replication and the G1/S cell cycle transition. In contrast, when bound to Cyclin A, Cdk2 phosphorylates Cdc6, HIRA, Skp1 and Cdc20. This activity in turn, prevents the re-replication of the genome and promotes the S/G2 cell cycle transition.

JNK1α1

JNK1 belongs to a group of kinases that has been implicated in multiple cellular processes such as cell survival, proliferation, embryonic development, T cell activation and differentiation (reviewed in (*130*). Activated by environmental stress and inflammatory cytokines, the JNK group of kinases is encoded by three genes that produce ten isoforms due to alternative splicing. Splice variants differ in their C-terminal extension length and substrate binding motif. This produces ten substrate specific isoforms that are either 46 or 54 kDa. The JNK isoform studied in this work is JNK1α1 (JNK1 *gene*; α *substrate motif*; 1 *short C-terminus*). Activation of JNK is carried out by the phosphorylation of the TPY motif in its activation loop by MKK4 and MKK7. Activated JNK dimerizes and translocates to the nucleus where it phosphorylates the

transcription factors c-Jun, JunB, JunC, ATF2, and NFAT4. These events then result in altered gene expression allowing the cell to adjust to its new environment. The structure of JNK is relatively simple with no large defined regulatory domains flanking the kinase core much like Cdk2. However, according to the JNK3 crystal structure, the JNK group differs in that they contain an extensive C-terminal region that wraps around the kinase domain interacting with portions of the αE-helix and the entire αC-helix (*131*). Another structural feature that distinguishes the JNK group is the presence of two additional anti-parallel β-sheets that precede motif I, which help to form a β–sandwich within the N-terminal lobe. These added structural features no doubt provide JNK with added stability and may account for why JNK has not been shown to be an Hsp90 client.

Protein Kinase Interactions with the Hsp90 Chaperone Machine

As previously explained, protein kinases are highly involved in initiating and regulating almost every cellular process in eukaryotic cells, and many of these kinases are dependent upon the Hsp90 chaperone machine to function properly. Despite its being over a decade since discovering the relationship between kinases and Hsp90, the basis by which certain protein kinases are recognized, recruited, and folded by Hsp90 is still not completely understood.

What is understood about the recognition of kinases by Hsp90 and Cdc37, is that they predominantly interact with the catalytic domain of each client kinase. The specifics of their interactions, however remain a mystery. Deletion mutagenesis of the protein kinases Akt and PDK1 initially led to the hypothesis that motifs IV-VIII were only needed for interaction with Hsp90 (*132, 133*). While this data indicated that motifs IV-

VIII were necessary for interaction with Hsp90, it did not indicate if this region was sufficient for Hsp90 interaction. This question was then addressed by our group, using deletion mutagenesis and puromycin-treated polyribosome pop-off experiments to study the interactions of both Hsp90 and Cdc37 with Lck and the heme-regulated eIF2α kinase, HRI (*108*). This work subsequently demonstrated that motifs V-VIII are not sufficient for Hsp90 and Cdc37 binding when expressed by themselves, but are indeed necessary when expressed in a construct containing either a complete N-terminal or C-terminal kinase lobe.

Our data suggested that since motifs V-VIII span both the NL and CL and do not form a stable multilayered structure, that Hsp90 and Cdc37 do not recognize unstructured polypeptides containing little to no tertiary structure (*108*). If Hsp90 and/or Cdc37 did recognize this unstructured polypeptide it would then suggest that recognition is based on amino acid sequence. This is most unlikely since the amino acid sequence of these structural motifs is not highly conserved among client kinases and may even contain the insertion of regulatory domains between the NL and CL, such as found in HRI. Thus, this indicates that a complete lobe (either NL or CL) must be present along with motifs V-VIII, in order for there to be enough tertiary structure and/or domain stability for Hsp90 to recognize and bind the protein kinase construct. Thus, these observations have led to the hypothesis that Hsp90 and Cdc37 binding is driven by tertiary structure recognition.

The subsequent chapters in this thesis primarily are focused on experiments carried out to characterize the interactions between Hsp90/Cdc37 and protein kinases: the molecular basis of kinase recognition by Hsp90 and Cdc37. As well as, why are some

protein kinases clients of Hsp90 and Cd37 while others are not? And structurally, what might be the difference between these types of kinase?

CHAPTER II

Definition of Protein Kinase Sequence Motifs that Trigger High Affinity Binding of Hsp90 and Cdc37

Introduction

In vivo, Hsp90 function is essential for the biogenesis and support of numerous cellular proteins that regulate signal transduction, including transcription factors, protein kinases, and a wide variety of proteins, such as nitric oxide synthase and telomerase [reviewed in (*15, 134, 135*)]. Despite over a decade of intense study, the basis for Hsp90's recognition of its diverse clients remains one of the primary mysteries in the field. Studies using site-directed and deletion mutagenesis (*132, 133, 136-140*) have identified potential sites within steroid hormone receptors and protein kinases that may be recognized by Hsp90. However, while a recent study has indicted that point mutations generated in the Cdk4 kinase (*141*) affect its interaction with Hsp90, no common primary, secondary and/or tertiary structural motifs have been defined that may be responsible for Hsp90-cleint recognition.

Hsp90 functions in conjunction with a coterie of non-client co-chaperone partners (reviewed in (*15, 134, 135*)). Current models suggest that co-chaperones may confer specificity to Hsp90 facilitated protein folding by also interacting with client targets (*136, 142, 143*). Cdc37 is one such Hsp90 co-chaperone that interacts with immature forms of Hsp90-dependent kinases (reviewed in (*96*)). The biochemical activity of Cdc37 is essential for Hsp90-mediated support of kinase function [e.g., (*106, 111, 125, 144, 145*)].

In vitro, Cdc37 exhibits a "passive" chaperone activity similar to that of Hsp90.

Cdc37 has been shown to prevent the aggregation of denatured protein, while also being able to maintain the protein in a state that is competent to refold with no requirement for input of energy from ATP hydrolysis (*144*). Furthermore, the domain structure of Cdc37 has been determined, and its kinase-binding and Hsp90-binding activities have been mapped to its N-terminal and middle domains, respectively (*106, 110, 146*), and we have demonstrated that phosphorylation of Cdc37 on Ser13 by CK II is required for the ability of Cdc37 to bind protein kinases (*147*). However, while the specific basis for the recognition of protein kinases by Cdc37 is unknown, recent studies have mapped the recognition motif to the N-terminal lobe of the catalytic domain of protein kinases (*108, 126, 141*).

Hsp90 inhibitors have aided studies into the mechanisms underlying the function of Hsp90 and its co-chaperones (reviewed in (*15, 134, 135*)). Hsp90's N-terminal nucleotide binding domain is the site of action for the Hsp90 inhibitor geldanamycin (*37, 38, 148*), while its C-terminal domain contains a binding site for novobiocin, which also inhibits Hsp90 function (*66-68, 149*). Analyses with these compounds, with nucleotides and nucleotide analogs, and with site-directed mutants that alter Hsp90's ATP binding and/or ATPase activity have revealed that Hsp90 function is regulated via the binding and hydrolysis of ATP, which modulates the switching of Hsp90 between at least three alternative conformations (*15, 68, 134, 135*). In the presence of geldanamycin, Hsp90 binds weakly to client kinases in a salt-labile fashion (*106-108*). These aberrant Hsp90 heterocomplexes indicate that nucleotide-modulation of conformational switching is required to generate high affinity interactions of Hsp90 and Cdc37 with protein kinases (*106-108*). Like geldanamycin, the anion molybdate inhibits Hsp90 function. However,

in contrast to geldanamycin this inhibition reflects the ability of molybdate to "lock" or freeze Hsp90-kinase complexes in high-affinity salt-stable complexes (*106-108*). Thus, molybdate causes the accumulation of Hsp90 complexes containing the Hsp90 co-chaperones p23, Cdc37 and an assortment of other "late" complex components, such as TPR motif-containing immunophilins (*46, 48, 63, 90, 106-108, 150, 151*). However, while molybdate stabilizes the normally labile interaction of Hsp90 with steroid hormone receptor clients (*48, 107, 108*), Hsp90's interactions with kinase clients are stable to high salt concentrations independent of molybdate freezing (*48, 106-108*). Thus, we have utilized the ability of kinase constructs to trigger molybdate-independent, high-affinity, salt-stable interactions with Hsp90 and Cdc37 to define motifs recognized by these chaperones (*108*).

In this report, we have systematically deleted secondary structural elements from the catalytic domain of the Hsp90-dependent Src-family protein tyrosine kinase Lck to increase the resolution of motifs recognized by Hsp90 and Cdc37. The data confirm that Cdc37 recognizes structures or sequences present in the N-terminal lobe of the kinase's catalytic domain. Furthermore, the motifs within the N-terminal and C-terminal lobes of the catalytic domain that are required to trigger nucleotide-dependent conformational switching of Hsp90 and molybdate-independent high affinity binding of Hsp90 and Cdc37 to kinase constructs overlap at the junction of the two subdomains.

Experimental Procedures

Plasmids. The coding sequences for protein domains and sub-domains were cloned by PCR into a modified pSP64T plasmid (*152*) as previously described (*108, 153, 154*). N-

terminally His-tagged versions of each domain were constructed. Sequences represented: the Lck NT-terminal domains (NT-SH2-SH3: residues 1-243); catalytic domain (Cat: residues 228-498); the N-terminal lobe (NL: residues 228-316) of the catalytic domain of Lck plus the linker region between the lobes (NL-linker: residues 228-336); the N-lobe of the catalytic domain (NL: residues 228-316); the C-terminal lobe (CL) of the catalytic domain of Lck plus the linker between the two lobes (linker-CL: residues 317-498); the C-terminal lobe of the catalytic domain containing motifs (*123*) VIa-XI (CL: residues 336-498); the NL through conserved kinase motifs VIII (NL-APE: residues 228-410); the NL through conserved kinase motifs VII (NL-β7/8: residues 228-384); the NL through conserved kinase motifs VIb (NL-β7: residues 228-375); the NL through conserved kinase motifs VIa and the β6 sheet region (NL- αE-Cap: residues 228-361); the NL through conserved kinase motifs VIa (NL-αE: residues 228-355); the regions of Lck's catalytic domain encompassing kinase motif II through the CL (β3-CL: residues 261-498); the regions of Lck's catalytic domain encompassing kinase motif III through the CL (αC-CL: residues 276-498); the regions of Lck's catalytic domain encompassing kinase motif IV through the CL (β4-CL: residues 294-498); and the regions of Lck's catalytic domain encompassing kinase motifs IV-VIb (αC-β7: residues 276-375). These dissections are represented graphically in Fig. 11.

Co-immunoprecipitations of chaperones with His-tagged kinase constructs. Lck constructs were cloned via NcoI/EcoRI into a modified pSP64T plasmid that coded for an N-terminal His_6-tag. Using a no DNA blank as control for non-specific binding (NS), each construct was synthesized and radiolabeled with [^{35}S]-Met by coupled

transcription/translation in 60 μl of nuclease-treated rabbit reticulocyte lysate (TnT, Promega) for 24 minutes at 30^0 C. Each sample was then split into two (30 μl) aliquots, supplemented with either 0.5 μl of 1 M sodium molybdate (final concentration 17 mM) or deionized water, and then incubated for another 1 min. All samples were then immediately placed on ice, clarified and immunoabsorbed with 25 μl of anti-His_5 antibody (Qiagen) bound to anti-mouse resin for 1 hour at 4^0 C (*108*). Immunoresins were then washed once with P50T (10 mM Pipes (pH 7.2), 50 mM NaCl, and 0.5% Tween-20), three times with P500T (same as P50T except with 500 mM NaCl), and again with P50T. No $NaMoO_4$ was present in the wash buffers. Finally, the samples were boiled in SDS sample buffer, separated on SDS-PAGE, transferred to PVDF membrane, and blotted for both endogenous Hsp90 and Cdc37 (*108*).

To load approximately equal molar amounts of each translation product, a portion of each TnT reaction was spotted on a piece of filter paper, and precipitated with 10 % trichloroacetic acid. After boiling the filters in 5% trichloroacetic acid, the filters were washed sequentially with ethanol, and acetone, and then dried. The filters were subsequently bleached with 15% hydrogen peroxide, dried and counted in a scintillation counter. Equivalent molar amounts of samples were loaded from SDS-elution of resins based on the amount of [^{35}S]-Met incorporated into protein construct in each TnT reaction and the number of methionine residues present in each construct. Samples were separated by SDS-PAGE, and transferred to PVDF membrane for Western blotting and autoradiography. Membranes were: stained lightly with Coomassie blue to visualize protein patterns and the molecular weight markers; dried and exposed to film to determine presence and pattern of radioactively labeled protein bands; and then cut

according to molecular weight markers for probing with the appropriate anti-chaperone antibodies. Immunoadsorption of each target kinase construct was confirmed by autoradiography (not shown in the figures).

Results

Characterization of the interactions of Lck's subdomains with Hsp90 and Cdc37. While Hsp90 and Cdc37 recognize and interact with the catalytic domain of protein kinases (*99, 106, 111, 132, 133, 155-157*), we have recently reported that Hsp90 also interacts with the N-terminal heme-binding domain of the heme-regulated eIF2α kinase, HRI (*108*). While the kinase domains of Src-family kinases retain the ability to bind Hsp90, the possibility that Hsp90 may have additional interactions with the N-terminal domains of this kinase family (i.e., the unique N-terminal, SH3 and SH2 domains) has not been investigated. Therefore, N-terminally His-tagged full length (fl) Lck, the N-terminal domains of Lck (NT-SH3-SH2), the Lck kinase catalytic domain (Cat) and the N-terminal lobe (NL) and C-terminal lobe of the catalytic domain that contained the sequences which links the two lobes (linker-CL) were cloned and expressed by coupled transcriptional/ translation in nuclease-treated reticulocyte lysate. The domains were then immunoadsorbed with anti-His-tag antibodies and analyzed by SDS-PAGE and Western blotting for co-adsorbing chaperones. Consistent with previous findings (*108*), full length Lck and Lck's catalytic domain formed salt-stable complexes with Hsp90 and Cdc37 independent of the presence of molybdate (Fig. 8). Hsp90 and Cdc37 also co-adsorbed with the NL construct of Lck, but molybdate was required to stabilize their interactions. Hsp90, but not Cdc37, interacted with the CL-linker construct of Lck and

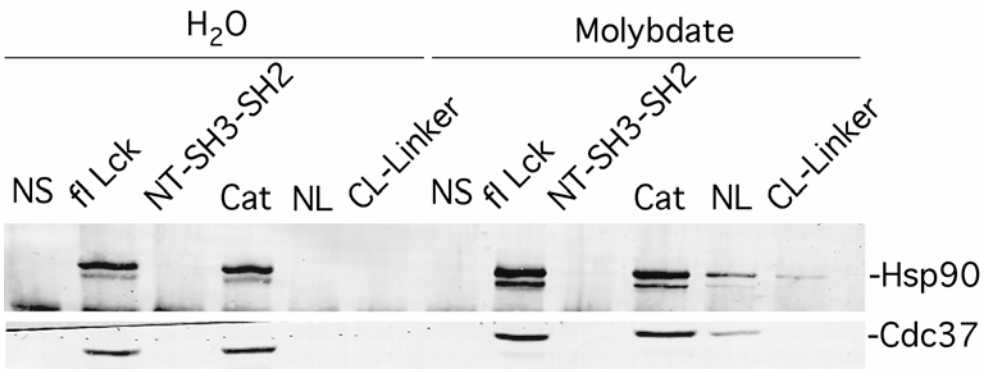


Fig. 8. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck.** ^{35}S-Labeled His-tagged Lck constructs (full-length (fl) Lck, the N-terminal domains of Lck (NT-SH3-SH2), the Lck kinase catalytic domain (Cat), and the NL and CL-linker constructs) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence or absence of 17 mM molybdate. Lysate containing no template (NS, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by Western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures." Top panel, Western blotted with anti-Hsp90; bottom panel, Western blotted with anti-Cdc37 antibodies.

molybdate was also required to stabilize this interaction to washing with high salt buffer. No Hsp90 or Cdc37 was found to coadsorb with the NT-domains of Lck (NT-SH3-SH2) in the presence or absence of molybdate. Thus, the interaction of Hsp90 and Cdc37 with Lck was confined to its catalytic domain, and, as we have previously observed, the interaction of Cdc37 was localized to the NL of Lck (*108*).

Definition of the minimal motifs within the C-lobe of Lck's catalytic domain required for molybdate-independent high-affinity binding of Hsp90 and Cdc37. To further delineate the kinase segments that interacted with Hsp90 and Cdc37, we utilized the crystal structure of Lck's kinase domain as a guide to express constructs from which conserved kinase sequence motifs were deleted from the C-terminal lobe of the kinase (Figure 9). The Lck constructs containing the NL and deletions that terminated the catalytic domain just after the "APE-box" (motif VIII, N-APE), the conserved DFG residues (motif VII, NL-β7/8) and the conserved HRDL(K/R)xxN residues (motif VIb, NL-β7) formed complexes with Hsp90 and Cdc37 that were stable in high salt in the absence of molybdate, and the binding occurred at levels that were nearly equivalent to the binding of the catalytic domain (Fig. 9A). The Lck construct that contained the NL and sequences from the linker region terminating with the α-D-helix that connects the two kinase lobes (motif V, NL-linker) showed a very weak salt stable interaction with Hsp90 and Cdc37, which was markedly enhance in the presence of molybdate. While again as shown in Figure 8, Hsp90 and Cdc37 co-adsorbed with the NL, but only in the presence of molybdate.

To more finely characterize sequences within the C-terminal kinase lobe of Lck that were required to trigger the high affinity binding of Hsp90 and Cdc37, two more

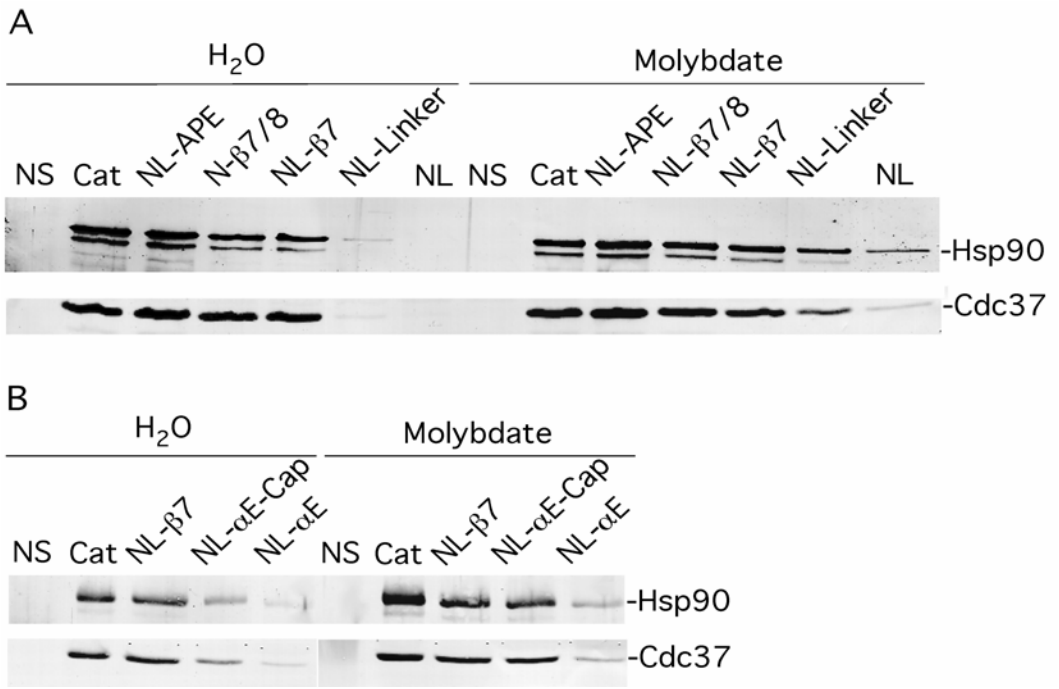


Fig. 9. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck catalytic domain containing deletions of CL kinase motifs. (A)** ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (Cat), NL-APE, NL-β7/8, NL- β7, and NL-linker) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence or absence of 17 mM molybdate. **(B)** ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (Cat), NL-β7, NL-αE-Cap, and NL-αE) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence or absence of 17 mM molybdate. Lysate containing no template (NS, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by Western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures."

constructs containing the NL and a portion of the CL were constructed: an NL with a deletion terminating prior to the catalytic Asp residue contained in motif VIb (NL-αE-Cap); and an NL with a deletion terminating at the end of the αE-helix which lacks the three amino acids that cap the C-terminal end of this helix [equivalent to the β-6 sheet in the nomenclature for the cAMP-activated protein kinase (*123*)] and likely stabilizes its structure (NL-αE). The NL-αE-Cap construct showed molybdate-independent salt-stabile interactions with Hsp90 and Cdc37 that were reduced by approximately 50% compared to the Lck catalytic domain and the NL-β7 construct (Fig. 9B). However, in the presence of molybdate, the binding of Hsp90 and Cdc37 to the NL-αE-Cap construct was nearly equivalent to the binding of the proteins to the NL-β7 construct. In contrast, the interaction of Hsp90 and Cdc37 with the NL-αE construct was scarcely detectable in the absence of molybdate, and was markedly decreased even in the presence of molybdate when compared to the NL-αE-Cap construct. Thus, it seems that the six amino acid residues just past the end of the α-E-helix in the C-terminal lobe of Lck's catalytic domain appear to be required to trigger Hsp90 to adopt its high affinity binding conformation.

Definition of the minimal motifs within the N-lobe of Lck's catalytic domain required for molybdate-independent high-affinity binding of Hsp90 and Cdc37- Similarly, to more clearly characterize sequences within the N-terminal kinase lobe of Lck that were required to trigger the high affinity binding of Hsp90 and Cdc37, we prepared constructs of Lck's catalytic domain that contained the complete C-terminal kinase lobe (CL) from which conserved kinase sequence motifs were deleted from the N-terminal lobe. Constructs of the catalytic domain were made from which: the β1 and β2

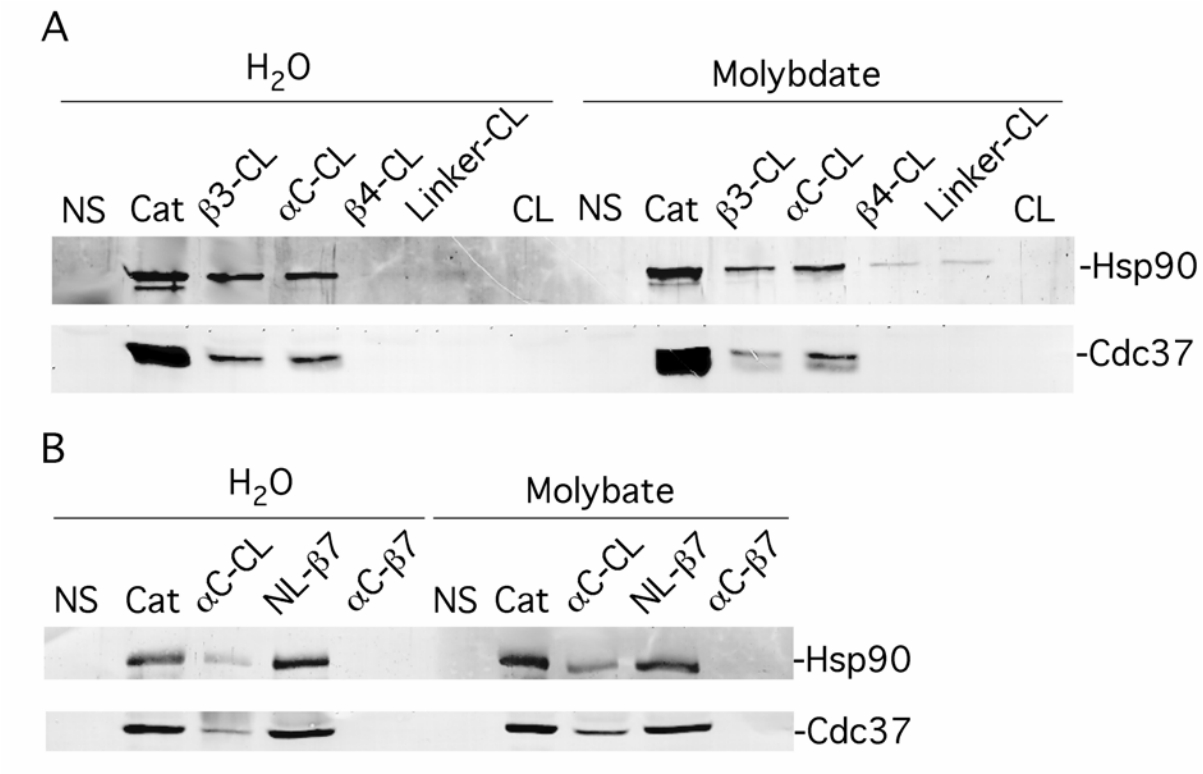


Fig.10. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck catalytic domain containing deletions of NL and CL kinase motifs. (A)** ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (Cat), β3-CL, αC-CL, β4-CL, linker-CL, and CL) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence or absence of 17 mM molybdate. **(B)** ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (Cat), αC-CL, NL-β7, and αC-β7) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence or absence of 17 mM molybdate. Lysate containing no template (NS, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures."

sheets (motif I) were deleted (β3-CL); the β1, β2, and β3 three sheets (motifs I-II) were deleted (αC-CL); the region from the β1 sheet through the α-C-helix was deleted (motifs I-III, β4-CL); the region from the β1sheet through the β5 sheet was deleted (motifs I- IV, linker-CL) and the region from the β1 sheet through the connecting strand including the α-D-helix was deleted (motifs I-V, CL). Of these constructs, the β3-CL and the αC-CL constructs showed molybdate-independent salt-stable interactions with Hsp90 and Cdc37 that were reduced by approximately 50% compared to the Lck catalytic domain (Fig. 10A). The β4-CL, linker-CL and the CL constructs showed no interaction with Hsp90 or Cdc37 in the absence of molybdate. In the presence of molybdate the β4-CL and the linker-CL constructs were found to have a very weak interaction with Hsp90, but no interaction with Cdc37. The CL construct showed no interaction with either Hsp90 or Cdc37 in the presence of molybdate. Thus, the presence of the α-C-helix within in the N-lobe of the Lck kinase was required for the binding of Cdc37. In addition the region of Lck that connects the N- and C-lobes of the catalytic domain is required for minimal recognition by Hsp90, and the presence of molybdate is required to stabilize this interaction.

Determination of the Effect of the Region that Links the N- and C-terminal Kinase Lobes on Hsp90/Cdc37 Binding. To test the hypothesis that the borders required for Hsp90 and Cdc37 that were identified in the experiments shown in Figures 9 and 10A defined the minimal Hsp90/Cdc37 interacting motif a construct of Lck was made that contained the region between the α-C-helix and β7 sheets (motifs III-VIb, αC-β7). While the αC-CL and the NL-β7 constructs demonstrated molybdate-independent salt-stable binding to Hsp90 and Cdc37, albeit at a somewhat reduced level, no interaction of

the αC-β7 construct was observed with Hsp90 and Cdc37, even in the presence of molybdate (Fig. 10B).

Discussion

The work presented here further defines the motifs or structures that are required for recognition of kinases by Hsp90 and Cdc37, as defined by molybdate-stabilized binding of Hsp90 and Cdc37 to kinase constructs, and for triggering conformational switching of Hsp90, as defined by salt-stable binding of Hsp90 and Cdc37 to kinase constructs that occurs independent of the presence of molybdate. Data presented here indicate that the Hsp90-Cdc37 heterocomplex binds to kinase molecules primarily via the specific recognition of kinase motifs or structures present in both the N-terminal and C-terminal lobes of the kinase catalytic domain of Lck. This is consistent with our previous work that demonstrate that multiple kinase motifs present within both lobes of kinases are required to trigger molybdate-independent high-affinity binding of Hsp90 and Cdc37 to protein kinases (*108*).

The linker region that connects the N- and C-terminal lobes of the Lck kinase appears to play a critical role for Hsp90 binding. The NL-linker and the linker-CL both interacted with Hsp90 albeit with reduced affinity. Stabilization of this interaction of Hsp90 with these constructs by the addition of molybdate markedly increased the level of hsp90 binding indicating that the minimal constructs were deficient in triggering Hsp90 conformational switching. The NL and CL constructs that lacked the linker region failed to bind Hsp90 in the absence of molybdate, and only the NL exhibited any detectable

interaction with Hsp90 (~5% compared to the catalytic domain control) in the presence of molybdate.

Results presented here together with previously published data (*108, 141*) localize the Cdc37 recognition motif to the αC-helix and the αC-β4 loop motif in the N-lobe of the catalytic domain of protein kinases. Cdc37 did not interact with the linker-CL Lck construct, while Hsp90 bound the construct. Lck constructs of the CL containing deletions of the β1, β2 and β3 sheets within the N-lobe stably bound Cdc37 and Hsp90 in a molybdate-independent manner, although the level was reduced by ~50% compare to the intact catalytic lobe. Deletion of the αC-helix negated the interaction of Cdc37, but not Hsp90 with Lck constructs containing the complete CL. Previous work investigating the interaction of Cdc37 with Cdk4 constructs fused to the C-terminus of GST indicated that GST-Cdk4 constructs containing the NL of Cdk4 through the αC-helix and residues within the loop between the αC-helix and β4 sheet bound Hsp90 and Cdc37 (*141*). Deletion of residues in the loop caused a marked reduction in Hsp90/Cdc37 binding. Thus, the results of the two studies intersect at the region of the αC-helix-β4 loop.

The presence of a β-sheet structure appears be necessary for recognition of the N-lobe of kinase catalytic domains by Hsp90 and/ or Cdc37. However, our results differ from those obtained in the studies utilizing the Cdk4 kinase (*141*). Single layered β-sheet structures are not stable, as the hydrophobic face of the sheet in solution would be exposed to water. Thus, β-sheet structures must interact with an additional "layer" for their structure to be stabilized. The crystal structure of Cdk2 [the protein kinase with the greatest sequence similarity to Cdk4 that has been crystallized

(*129*)] suggests that there is no interaction between the hydrophobic face of the first three strands of β-sheet in the kinase N-lobe and the hydrophobic face of the αC-helix. However, the crystal structures of the inactive conformations of c-Src and Hck indicate that their β4/5-sheets may be stabilized through interactions with conserved hydrophobic residues in the linker region that connects the SH2 domain to the catalytic domain (*158-161*). Thus, Hsp90 and/ or Cdc37 may also act to stabilize the β-sheet structure within the N-lobe during folding in the absence of other interactions that stabilize the domain in the mature structure.

Thus, the results obtained in this study utilizing the Lck kinase catalytic domain compared to the results obtained with Cdk4 kinase suggest that there are subtle differences between protein kinase families relative to how their five stranded β-sheet layers are stabilized during kinase folding, in addition to how they are packed onto and influence the orientation of the αC-helix. Subtlies in the topology of N-lobe β-sheet layers likely account for the observation that mutation of Gly15 in the P-loop in the Cdk4 kinase or deletion of the first two strands of the β-sheet markedly decreases the binding of Cdc37, while deletion of the entire P-loop structure of the Lck catalytic domain has no effect on the binding of Hsp90 and Cdc37 to the β3-CL Lck construct. Since Cdc37 functions to foster the active conformation of protein kinases, the interactions of Hsp90/Cdc37 with components of the N-lobe may function to stabilize the β-sheet structure in a manner that facilitates Cdc37-induced rotation of the αC-helix. The crystal structures of the inactive conformations of Cdk2, Src and Hck indicate that the catalytic Glu in the αC-helix lies on the outer surface of the proteins and that the helix must be rotated to properly position of the Glu within the catalytic pocket (*129, 158-161*).

However, the crystal structures suggest that interactions between residues within the αC-helix and β4-loop with residues present in the C-lobe would sterically impede or prevent such a rotation from occurring spontaneously.

Our data indicate that the minimal kinase structure that is required to trigger molybdate-independent high-affinity binding of Hsp90/Cdc37 is one complete lobe of the protein kinase's catalytic domain, the connecting linker and a portion of the second lobe. For the constructs containing the complete C-lobe of Lck, the minimum N-lobe structure consists of the αC-helix and the β4/5-sheets, while for the complete N-lobe the minimum C-lobe structure consists of the αE-helix and the residues that cap its structure at its C-terminus (equivalent in kinase nomenclature to the β6 strand of the cAMP-activated protein kinase (*123*)). Although it is noteworthy that inclusion of catalytic residues that follow the β-7 sheet in the C-lobe restore nearly wild-type binding of Hsp90 and Cdc37 to construct of Lck's NL. However, the combination of these minimum structures, the construct containing the α-C-helix through the αE-helix-cap (αC-β7) does not bind Hsp90 or Cdc37. This finding is consistent with observation that substantial or partial folding of client proteins appears to occur prior to recruitment of Hsp90 to the client. Thus, we have further defined the minimum motifs within protein kinase clients that are recognized and "trigger" the Hsp90 chaperone machine to undergo its nucleotide-mediated conformational switching. Furthermore, the results indicate that the presence of the minimal Cdc37 recognition motif is not sufficient to stabilize the binding of Cdc37 to the construct in the absence of a stabile interaction of the construct with Hsp90.

The localization of this "switching motif / conformational trigger" is also noteworthy as the minimal structures within the two domains intersect at the region that

makes up the “hinge” between the two lobes of the kinase (*162*). With the exception of the “activation-loop”, comparison of crystal structures of inactive and active conformations of protein kinases indicate that the position of residues contained within the C-terminal lobe of protein kinases are nearly superimposable. Additionally, this analysis of kinase structures indicates that the αC-helix-β4 loop and the peptide strand connecting the N-terminal and C-terminal catalytic lobes act as a key region for global changes in kinase conformation that regulate the activation and activity of protein kinases (*162*). Our data suggest that these kinase motifs are critical for the binding of Hsp90 and Cdc37 to kinases. Furthermore, the superimposition of kinase structures indicate that the αC-helix of the N-lobe is rather randomly disposed, but with structural elements meeting at the point of the "hinge" (*162*). This region is located where the αE-helix intersects with the loop between the αC-helix and the β4-strand of the N-lobe and the linker region between the two kinase lobes. At this point the β7/8-strands, which contain the catalytic kinase residues present in the C-lobe, loop over the αE-helix and pass between the loop connecting the αC-helix and β4-strand and the strand that connects the two kinase lobes (Fig. 14A). Premature packing of the αC-helix-β4 loop and the peptide strand connecting the two catalytic lobes onto the αE-helix would preclude the proper positioning of the β7/8-strands. Thus, the Hsp90 chaperone complex may function to allow the independent folding of the two catalytic subdomains of protein kinase prior to their subsequent packing into an active or activatable structure. This hypothesis would be consistent with the apparent requirement for at least one full kinase subdomain and additional sequences within the second subdomain, and that the minimal motifs required

for triggering high affinity binding of Hsp90 intersect at the point at which the two subdomains interact.

Comparisons of crystal structures indicate that twisting motions about the hinge affect the relative position of the N-terminal kinase lobe and the αC-helix. This motion affects the position of the conserved catalytic Lys and Glu residues relative to the catalytic residues present in the C-terminal lobe (*162*), such that small perturbations of these structural elements have significant effects on kinase conformation, and kinase activity (*162*). Furthermore, in the absence of bound nucleotide, the hydrophobic pocket that is occupied by the adenine ring may collapse upon exposure of the ATP binding pocket to water. Thus, the Hsp90 chaperone machinery may function to correct structural perturbations that render the catalytic domain inactive by physically separating the two subdomains of a kinase's catalytic lobe allowing them to refold independently. Concurrently, Cdc37 might function to stabilize the orientation of the catalytic Glu residue in the αC-helix relative to the position of the catalytic Lys. Subsequently, the kinase subdomains would then be released from the Hsp90/Cdc37 complex and allowed to interact (repack) into an active or activatable conformation that is capable of binding ATP.

What mechanisms then govern Hsp90's recognition of protein structures and its switching from low affinity to high affinity binding? The data of Pratt and co-workers (*137*) suggest a model of the ligand-binding domain (LBD) of the glucocorticoid receptor acting as a "hinged pocket". They propose that Hsp90 recognizes α-helical structures that interact at the interface between the two subdomains of the LBD, and that sequences

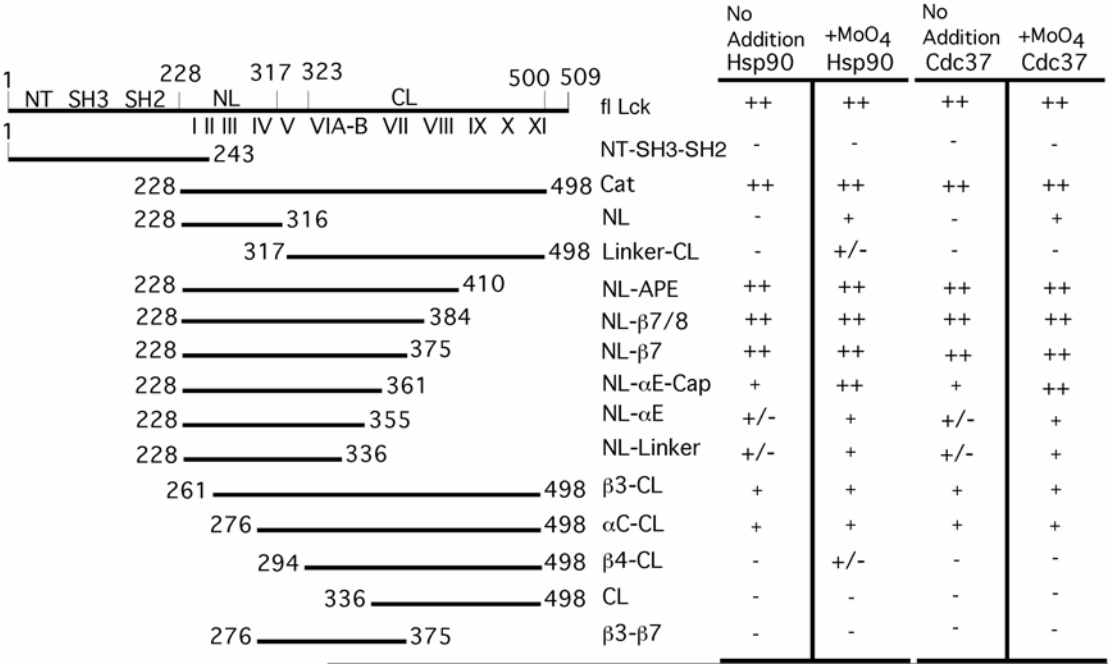


Fig. 11. **Summary of protein kinase constructs used for analysis of Hsp90 and Cdc37 binding to Lck constructs.** The domain architecture of the Lck kinase is indicated at the top of the diagram. The numbers above the lines indicate the amino acids residues that flank: the unique N-terminal domain of Lck (NT), SH2 and SH3 domains, the NL of Lck, the linker region between the NL and CL of Lck, the CL of Lck, and the C-terminal tail of Lck. The Roman numerals below the line indicate the locations of conserved kinase motifs I-XI. The lower lines indicate the amino acid residues present in each of the domain and subdomain constructs studied in this report and where the constructs begin and terminate with the abbreviation used to refer to each construct indicated to the right of each line. The chart to the right summarizes the binding of Hsp90 and Cdc37 to the constructs in the absence or presence of molybdate: ++, binding equivalent or nearly equivalent to the catalytic domain; +, binding reduced by ~50% relative to the catalytic domain; +/-, binding less than 10% of that of the catalytic domain; -, no detectable binding

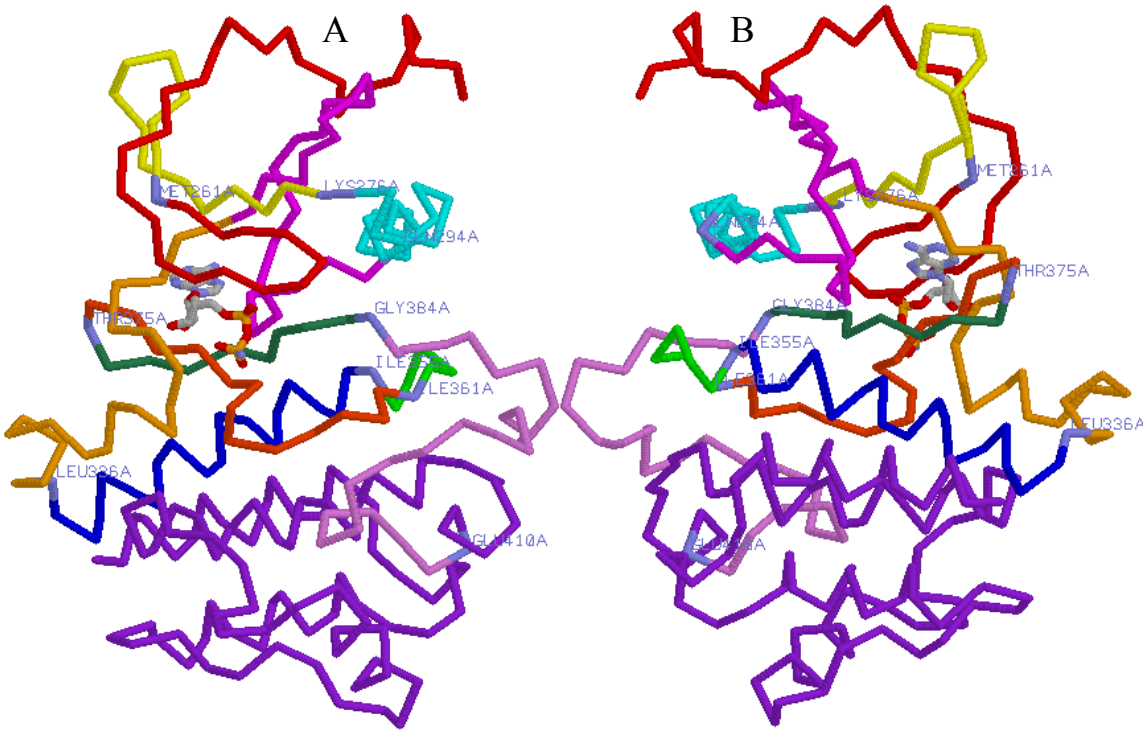


Fig. 12. **Depiction of Lck deletion mutant constructs.** A different color is shown for each segment of Lck deleted in respect to **Fig. 11**: residues 228-261, red; residues 262-276, yellow; residues 277-294, cyan; residues 295-316, magenta; residues 317-336, orange; residues 337-355, blue; residues 356-361, green; residues 362-375, redorange; residues 376-384, greenblue; residues 385-410, violet; residues 411-498, purple. The kinase domain is shown with its ATP binding cleft facing either **(A)** toward or **(B)** away from the viewer.

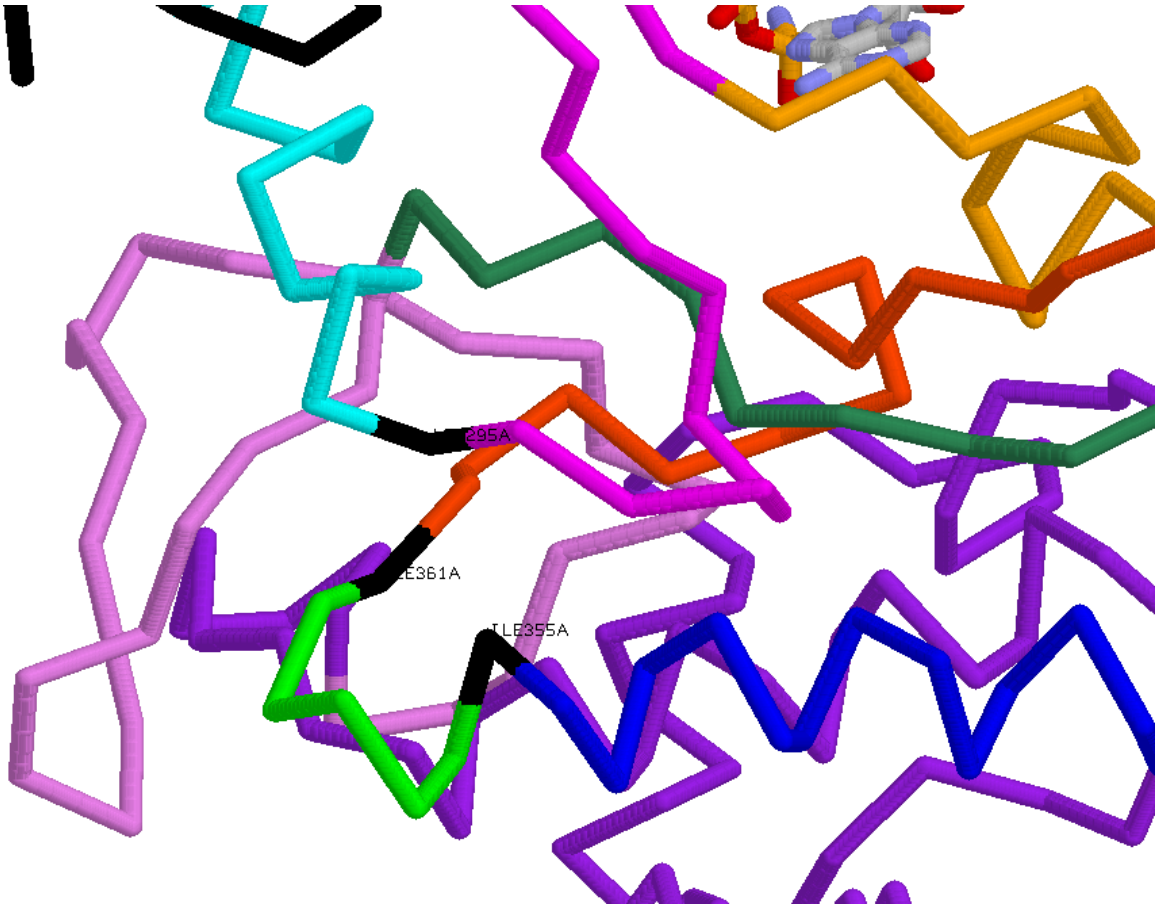


Fig. 13. **Close-up of the αC-helix-β4 loop interface with the αE-helix and the αE-helix capping motif.** The αC-helix and the β4 loop are shown in cyan and magenta, respectively. The αE-helix and the αE-helix capping motif are shown in blue and green, respectively. Deletion sites are designated and labeled black.

present in a β-sheet structure function as a hinge between the two subdomains. This interaction of Hsp90 with the LBD is proposed to break the hydrophobic clasp formed by the interaction between the two subdomains, allowing hormone to access its ligand binding site. The trypsin sensitivity of the LBD near the β-stranded region suggests that its flexibility would allow separation of the two subdomains (*137*). Similar to the findings reported here, deletion analysis of the LBD (*143*) indicates that a construct containing the complete N-terminal subdomain of the LBD and the β-stranded hinge through the first α-helix of the C-terminal portion of the ligand binding subdomain is the minimal construct of the glucorocorticoid receptor that binds Hsp90 (Fig. 14B).

Of further interest is the observation that the two strands of β-sheet that make up this proposed hinge are a single layer motif with the sequence 620-LLCFAPDLII-629. The side of the sheet facing the ligand binding pocket is composed exclusively of hydrophobic amino acids, with the surface of the sheet also having considerable hydrophobic character. The "hole" representing the ligand binding pocket results in both sides of the β-sheet being exposed to solvent. Thermodynamics might favor the collapse of the β-sheet structure into the protein's interior in the absence of hormone occupying its binding pocket, considering that this region has been reported to be conformationally mobile (*137*). Thus, an additional consequence of Hsp90 separating the two subdomains of the LBD would be to reposition the β-strands, and allow refolding of the hormone-binding pocket.

The findings of Pratt and co-workers, together with the data presented here suggest a common theme for Hsp90 function. Hsp90 and its associated co-chaperones may function to facilitate protein folding by breaking interactions between subdomains

within a protein's structure. Inspection of the crystal structures of Lck's kinase domain and the GR's LBD indicate that the structures of the flexible linker regions between the N- and C-terminal subdomains of each of these proteins, and the α-helical region that follows are strikingly similar (Fig. 14, right panels). The separation of the subdomains of SHRs or protein kinases may function to displace protein structures that impede the ability of critical amino acid residues to be properly positioned, or to reposition critical amino acids, which would subsequently allow the protein to attain its active or activatable (regulatable) conformation: attain a structure capable of binding ATP or hormone, respectively. For the *de novo* folding of protein kinases, Hsp90 may be required to reorient a two-stranded β-sheet structure, subsequently allowing the structure to occupy a "hole" formed at the junction between the two catalytic subdomains and for the ATP binding pocket to be properly folded. While for steroid hormone receptors, Hsp90 would function to reorient a two-stranded β-sheet structure, removing the structure from a "hole" which exists due to the absence of hormone occupying its LBD, subsequently allowing the unoccupied steroid hormone binding pocket to reform. These distinct variations between protein kinases and steroid hormone receptors may then account for the differences in the characteristics of their interactions with Hsp90. Whereas the catalytic domains of protein kinases can form high-affinity salt-stabile complexes with Hsp90 in the absence of molybdate (*107, 108*), the LBD of steroid hormone receptors require molybdate to stabilize Hsp90 binding (*89*). Additionally, while Cdc37 is required for Hsp90 to form high-affinity complexes with protein kinases and for their productive folding, Hsp90 has not been documented to require such a cohort when restoring steroid hormone receptor function.

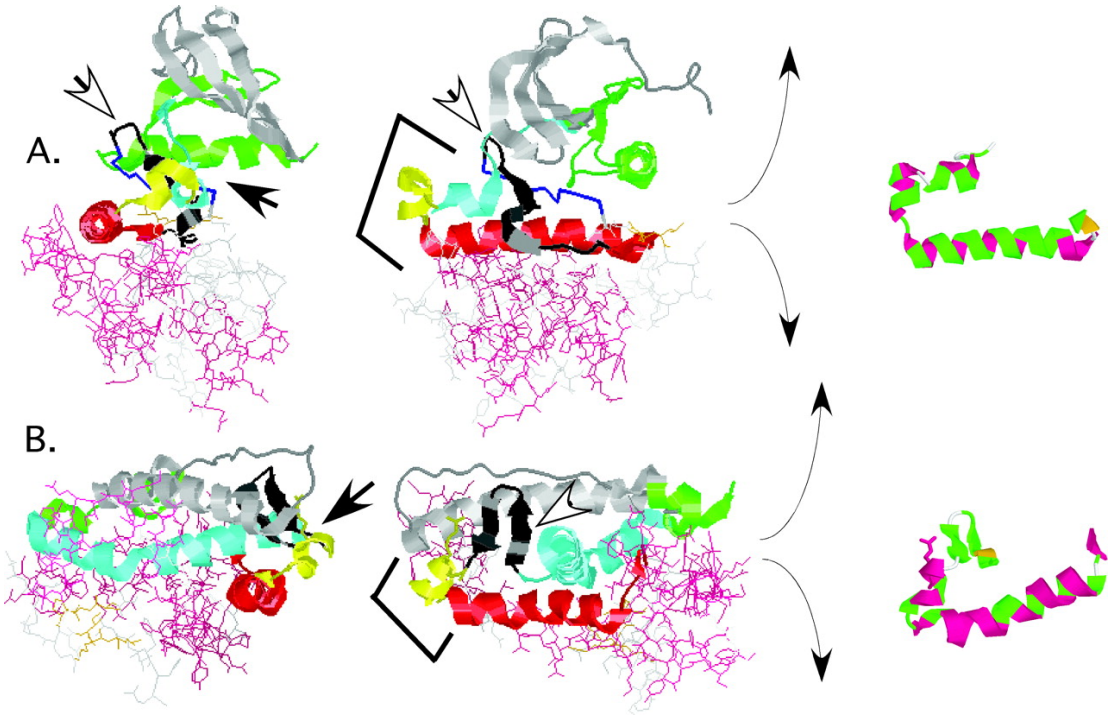


Fig. 14. **Diagrams of the Lck kinase catalytic (A) and the glucocorticoid receptor ligand-binding domain (B) highlighting structural motifs that have been implicated in their interactions with Hsp90.** The diagrams of Lck catalytic domain (PDB: 1QPC) and glucocorticoid receptor-LBD (PDB: 1NHC). **(A)** left and center panels, residues 231-275 (the first three β-strands of the Lck catalytic domain) are colored gray; residues 276-317 (the αC-helix and β-strands 4/5) are colored green; residues 318-329 (the random coil and α-D-helix) are colored cyan; residues 330-336 (the αhelical insert between the αD and αE-helix) are colored yellow; residues 337-361 (the αE-helix and its cap) are colored red; residues 362-375 (motif VIB containing the β-7 sheet) are colored black; the backbone of residues 376-384 (motif VII containing the β-8 sheet) are colored dark blue; and the remainder of the molecule is shown in wire frame mode and colored by structure (magenta, α-helix; white, random coil). **(B)** left and center panels, residues 530-537 (the α1-helix) and residues 582-587 (a short α-helix between helix 3 and 4 of the glucocorticoid receptor-LBD that are proposed to be part of the "hydrophobic clamp") are colored green; residue 538-581 (the end of the α1-helix through the α3-helix) are colored gray); residues 588-619 (the α4 and α5-helices) are colored cyan; residues 620-630 (the two strands of β-sheet in the linker region) are colored black; residues 631-638 (the α6 helix in the hinge region containing Arg^{633} shown in stick format) are colored yellow; residues 639-659 (the α7-helix) are colored red; the remainder of the C-terminal subdomain is shown in wire frame format and colored by structure (magenta, α-helix; yellow, β-sheet; white, random coil). **(A)** and **(B)**, right panels, cartoon diagram of the proposed hinge regions of the kinase domain of Lck and the LBD of the glucocorticoid receptor followed by the minimal part of the C-terminal subdomain of each molecule that is required for Hsp90 binding colored by structure, with hydrophobic residues colored green. **(A)** left and center panels, open arrows, positions of β7 and β8 strands that must past between the junction of the N- and C-terminal kinase subdomains. **(A)** and **(B)**, left panel, closed arrow, and center panel, open sided box, positions of linker regions between the two subdomains of each protein; curved arrows,indicate proposed movement of the subdomains that would be required to reposition the β-sheet structures at the interface between the two subdomains of each protein. **(B)** open arrow, position of the ligand-binding pocket behind the two strands of β-sheet.

CHAPTER III

Hsp90 and Cdc37 Interactions with the Protein Kinase Cdk2

Introduction

Hsp90 is a 90 kDa ATPase that functions as a molecular chaperone for a multitude of signal transduction proteins, which play critical roles in controlling practically every facet of a cell's physiology. The ability of Hsp90 to function as a chaperone to this wide array of signal transduction proteins is due, in part, to the coterie of non-client co-chaperones that are components of the Hsp90 heteromeric super-chaperone machine (*14, 15, 134, 135*). In this regard, the Hsp90 co-chaperone Cdc37 is a protein required for the viability of eukaryotic cells (*96, 97*). Cdc37 carries out a function essential for Hsp90's ability to support the activity of numerous protein kinases, as such Cdc37 is often referred to as the "kinase specific co-chaperone" (*106, 125, 144, 145*). However, Cdc37 has also been found in complexes with the androgen receptor and certain viral reverse transcriptases suggesting it likely has a broader set of clients (*112, 116*).

As noted above, protein kinases, which are one of the largest families of signal transduction proteins (*120*), often depend upon Hsp90 to carry out their function. Hsp90, together with Cdc37, interacts with protein kinases to facilitate their proper folding and/ or stabilize their structure (*106, 108, 125, 144, 145*). The productive interactions of Hsp90 and Cdc37 with protein kinases are known to require the presence of specific stuctural motifs within the protein kinase's bi-lobal structure (*108, 163*). Nevertheless, despite years of study, the exact manner of recognition of these structural motifs by Hsp90 and Cdc37 is still not completely understood.

Previous studies carried out by our laboratory, in which defined secondary structural elements were deleted from the Lck tyrosine kinase, have identified potential structural motifs required for high affinity binding of Hsp90 and Cdc37 to protein kinases (*108, 163*). However, our findings are at variance with studies done with the cyclin-dependent kinase, Cdk4 (*141*). In that work, the G-box (also known as P-loop) was identified as a motif needed for the interaction of Cdk4 with Cdc37, whereas in our Lck model the deletion of this motif had no effect on the binding of Cdc37 to Lck. Consequently, we have attempted to refine our understanding of Hsp90 and Cdc37 interactions with protein kinases by studying yet another kinase, the cyclin-dependent kinase Cdk2.

Cdk2 is a protein kinase that regulates cell cycle progression through the coordinated association with specific regulatory proteins, known as cyclins (*128*). Upon cyclin binding Cdk2 is able to undergo a conformational change that allows its activation loop to be phosphorylated on T^{160} by Cdk-activating kinase (CAK, also known as Cdk7/cyclin H) (*164*). Activated Cdk2 is then able to phosphorylate downstream effector proteins, which in turn help drive DNA replication and eventually S/G_2 transition. Additionally, Cdk2's kinase activity can be inhibited by phosphorylation of G-box residue Y^{15} by the bifunctional kinase Wee1 (*165-167*).

Previous genetic and yeast two-hybrid studies have shown that both Hsp90 and Cdc37 interact with Cdk2, suggesting that Cdk2 is an Hsp90 client kinase (*126*). Furthermore, it has been shown that Cdc37 interacts with the N-terminal lobe of Cdk2, an observation supported by our findings with Lck (*108, 163*). However, despite these and

other studies no true physical interaction between Cdk2 and Hsp90 or Cdc37 via pull-down assays has been documented (*125*).

In this report, we demonstrate that Cdk2 indeed physically interacts with Hsp90 and Cdc37 via pull-down assays and subsequently verify Cdk2 as a client protein kinase of the Hsp90/Cdc37 chaperone complex. A series of N-terminal and C-terminal deletions of Cdk2 were constructed to further define the structural motifs required to trigger molybdate-independent high affinity binding of Hsp90 and Cdc37 to the protein kinase. Our results again suggest that the αC-helix of Cdk2 is critical for the recognition of protein kinases by Hsp90 and Cdc37.

Experimental Procedures

Plasmids. Cdk2 constructs were cloned via NcoI/EcoRI into a modified pSP64T plasmid that coded for an N-terminal His_6-tag as previously described (*108*). N-terminally His-tagged versions of each domain were constructed. Sequences represented: full length Cdk2 (FL: residues1-298); the N-terminal lobe through structural motif VII (NL-β7: residues1-143); the N-terminal lobe through structural motif VI (NL-Cata: residues1-132); the N-terminal lobe through structural motifs V and part of VI or the uncapped αE helix (NL- αE: residues 1-120); the NL (residues 1-82); structural motif II through the CL (β3-CL: residues 19-298); structural motif III through the CL (αC-CL: residues 37-298); structural motif IV through the CL (β4-CL: residues 63-298). The plasmid, pcDNA3-CycA(wt)-AU5 which encodes for T7 driven Cyclin A with the C-terminal epitope tag, AU5 was a generous gift from Dr. Joan Ruderman of Harvard Medical School.

Synthesis of His-tagged Cdk2. Using a no DNA template as a control for non-specific binding, His-tagged Cdk2 was synthesized and radiolabeled with [^{35}S]-Met by coupled transcription/translation in 25 μl/ tube of nuclease-treated rabbit reticulocyte lysate (TnT, Promega) for 30 minutes at 30^0 C. For samples treated with drugs, geldanamycin (GA, 10 μg/ml final concentration) and dimethylsolfoxide (DMSO) were added prior to initiating synthesis.

Synthesis of Cyclin A with Cdk2 in the presence and absence of geldanamycin. Sp6 driven His-tagged Cdk2 and T7 driven Cyclin A-AU5 were synthesized separately and radiolabeled with [^{35}S]-Met by coupled transcription/translation in nuclease-treated rabbit reticulocyte lysate (TnT, Promega) for 30 minutes at 30^0 C in the presence of either 10 μg/ml GA or DMSO. Samples were then combined and mixed accordingly, 20 μl each to give a total volume of 40 μl, and incubated at 37^0 C for another 30 minutes.

Synthesis of His-tagged Cdk2 deletion constructs. Using a no DNA blank as a control for non-specific binding (NS), each construct was synthesized and radiolabeled with [^{35}S]-Met by coupled transcription/translation in 60 μl of nuclease-treated rabbit reticulocyte lysate (TnT, Promega) for 30 minutes at 30^0 C. Each sample was then split into three aliquots, two ~27 μl aliquots supplemented with either 0.5 μl of 1 M sodium molybdate (final concentration 17 mM) or deionized water, and then incubated for another 1 min. The remaining 5-6 μl of TnT lysate was put into 50 μl of SDS-sample buffer and ran on a 10% SDS-PAGE gel and checked by autoradiography as input controls.

Immunoabsorption of synthesized proteins. All immunoabsorption samples were immediately placed on ice, clarified by centrifugation with a Fisher 235c microfuge at 4^0 C for 5 min, and immunoabsorbed with 25-30 μl of either anti-mouse or anti-rabbit immuno-resin coupled with either mouse anti-His_5 (Qiagen), mouse non-specific IgG, rabbit anti-AU5 (Covance Research), rabbit sc-53 anti-PSTAIRE (Santa Cruz Biotechnology), or rabbit non-specific IgG for 1 h at 4^0 C. Immunoresins were then washed once with P50T (10 mM Pipes (pH 7.2), 50 mM NaCl, and 0.5% Tween-20), two times with P500T (same as P50T except with 500 mM NaCl), and again with P50T. No molybdate was present in the wash buffers. Finally, the samples were boiled in SDS sample buffer, separated on an 8% SDS-PAGE gel, transferred to PVDF membrane, and blotted for both endogenous Hsp90 and Cdc37 (*108*).

Cdk2 expression in GA treated K562 cells. K562 cells were grown in 6-well plates in RPMI 1640 media with 10% FBS and 1% antibiotics at 50% confluency. Cells were grown in the presence of either DMSO or 0.1 μM GA for 0, 12, 24, or 48 hours, then washed once with Hanks' balanced salts and lysed in lysis buffer: 0.5% Igepal, 20 mM Hepes pH 7.4, 100 mM NaCl, 2 mM EGTA, 10 % glycerol, and mammalian protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation and protein concentrations were determined via BCA assay (Pierce). Samples were then analyzed by SDS-PAGE and western blotting with polyclonal anti-Cdk2 (rabbit sc-748, Santa Cruz).

Results

Hsp90 and Cdc37 physically interact with Cdk2- Previous genetic studies have suggested that both Hsp90 and Cdc37 interact with Cdk2 (*126*). However, no direct physical interaction has been documented between Cdk2 and the Hsp90/Cdc37 chaperone complex. Indeed, one of the initial papers describing the interactions of Cdc37 with Cdk kinases concluded that Cdc37 did not interact with Cdk2 (*125*). Yet, Cdk4, a close relative of Cdk2, has been well established as client protein kinase that physically interacts with Hsp90 and Cdc37.

Complexes between Hsp90/Cdc37 and Cdk4 have been isolated from cell extracts, as well as reconstituted in vitro utilizing Cdk4 generated by coupled transcription/ translation (TnT) in rabbit reticulocyte lysate (*125, 141*). Thus, we tested whether a physical interaction between Cdk2 and Hsp90/Cdc37 could similarly be reconstituted in reticulocyte lysate with newly synthesized Cdk2. Cdk2 was cloned with an N-terminal His-tag and [^{35}S]-labeled His-tagged Cdk2 was synthesized by TnT in reticulocyte lysate. His-tagged Cdk2 was then immunoadsorbed with anti-His antibody and the immune pellets were washed at high stringency (buffer containing 0.5 M NaCl). Western blot analysis subsequently indicated that both endogenous Hsp90 and Cdc37 specifically co-absorbed with Cdk2 (Fig. 15A), consistent with results found for Cdk4.

Hsp90 inhibitors have aided studies into the mechanisms underlying the function of Hsp90 and its co-chaperones. The N-terminal nucleotide-binding domain of Hsp90 is the site of action for the inhibitor geldanamycin. In the presence of geldanamycin, Hsp90 is held in an "unlocked" conformation, which binds weakly to protein clients in a salt-labile fashion (*106-108*). To further test whether Cdk2 interacted with Hsp90 and Cdc37

in the manner of a protein client, His-tagged Cdk2 was expressed in the presence or absence of the Hsp90 inhibitor geldanamycin, immunoadsorbed and analyzed as described above. Again, consistent with the interaction of Hsp90 and Cdc37 with other client protein kinases (*106-108*), Hsp90 and Cdc37 did not form a complex with Cdk2 in the presence of geldanamycin that was stable to buffer containing 0.5 M NaCl (Fig. 15B). Therefore, these results support the hypothesis that Cdk2 is a client of the Hsp90/Cdc37 chaperone complex.

Antibody directed against the αC-helix of Cdk2 disrupts its interactions with Hsp90 and Cdc37- As noted above, in previously published work, it was concluded that Hsp90 and Cdc37 did not physically interact with Cdk2 (*125*). This work utilized the Cdk2-specific sc-53 antibody, which is directed against the amino acid sequence PSTAIRE in Cdk2's αC-helix. Since this work conflicted with the results described above, we tested whether Hsp90 and Cdc37 would co-absorb with newly synthesized His-tagged Cdk2 immunoadsorbed from reticulocyte lysate with sc-53, as opposed to anti-His-tag antibody. Western blotting indicated that Hsp90 and Cd37 co-adsorbed with His-tagged Cdk2 from reticulocyte lysate with anti-His tag antibody (Fig. 15C). Again this interaction was specific as no Hsp90 or Cdc37 was present in immune pellets containing bound mouse non-immune IgG.

Consistent with previous work, Hsp90 and Cdc37 did not co-absorb with His-tagged Cdk2 immunoadsorbed with sc-53. These results suggested that binding of sc-53 to sequences in the αC-helix prevents Hsp90 and Cdc37 from interacting with Cdk2. The binding of the sc-53 antibody to the αC-helix undoubtedly distorts Cdk2's conformation, and suggests that the αC-helix is recognized by, or is required

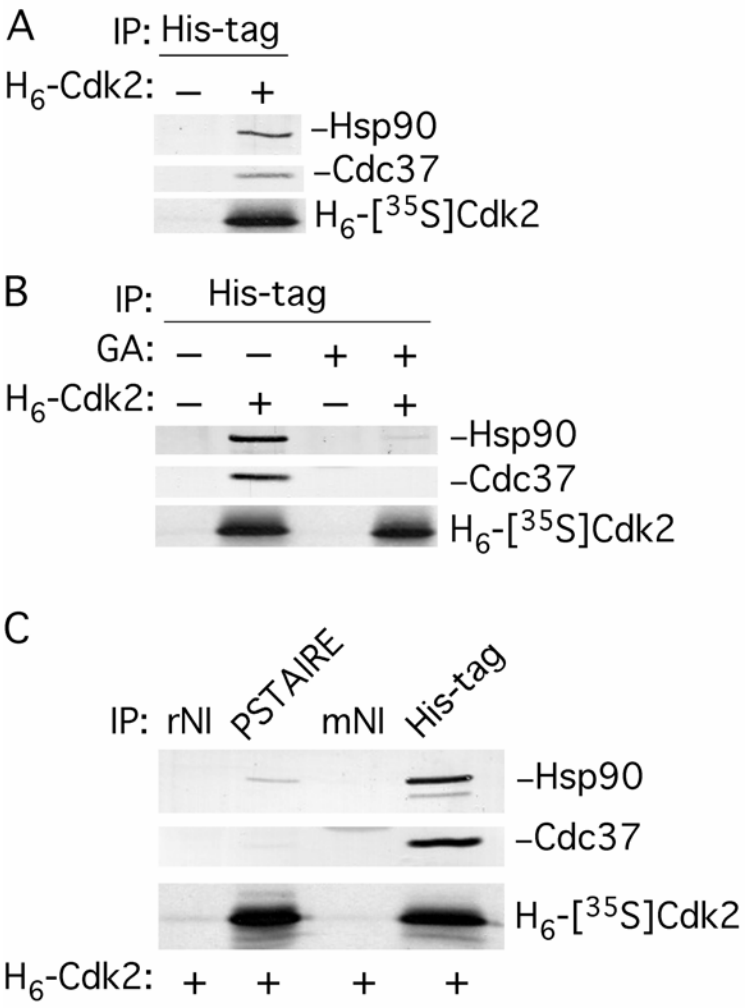


Fig. 15. **Interaction of Hsp90 and Cdc37 with newly synthesized Cdk2.** His-tagged Cdk2 was synthesized and labeled with [^{35}S]-Met in reticulocyte lysate for 30 min at 30^{0} C, followed by immunoabsorption. All samples were washed with P50T once, P500T twice, and P50T once more. Samples were then separated by SDS-PAGE and western blotted for co-absorbing endogenous Hsp90 and Cdc37, according to "Experimental Procedures". **(A)** Using a no DNA blank as a control for non-specific binding, His-tagged Cdk2 was immunoadsorbed with anti-His_5 coupled immunoresin. **(B)** Using a no DNA blank as a control for non-specific binding, His-tagged Cdk2 was synthesized in the presence and absence of geldanamycin (GA) and immunoadsorbed with anti-His_5 coupled immunoresin. **(C)** Using rabbit non-immune IgG (rNI) and mouse non-immune IgG (mNI) as controls for non-specific interactions, His-tagged Cdk2 was synthesized and specifically immunoabsorbed with anti-PSTAIRE (sc-53) and anti-H_5 antibodies.

to maintain the structure of motifs recognized by Hsp90 and/or Cdc37. This is result is consistent with our previous structural studies utilizing the Lck tyrosine kinase (*108, 163*), where Hsp90 and Cdc37 were able to interact with N-terminal deletion constructs that contained the αC-helix, but were unable to interact with the deletion constructs that were missing the αC-helix in the absence of molybdate. Together these results suggest that the αC-helix may play a critical role in the recognition and interaction of protein kinases with Cdc37 and Hsp90.

Inhibition of Hsp90 destabilizes Cdk2 in K562 cells- Inhibition of Hsp90 function *in vivo* destabilizes many Hsp90-dependent proteins and accelerates their proteolytic breakdown in cells (*23*). To further test the hypothesis that Cdk2 is an Hsp90-dependent kinase, we treated K562 myeloid leukemia cells with geldanamycin or DMSO (the drug vehicle control) for 0, 12, 24, and 48 hours. At the indicated times, cells were harvested, lysed in sample buffer and then frozen. Total protein concentration was determined via BCA assay, and equal amounts of protein from each cell lysate were analyzed by SDS-PAGE and western blotting. Little change in Cdk2 levels was observed at 12 hours (Fig. 16). However, at 24 hours the level of Cdk2 in geldanamycin treated cells was reduced by ~75% (Fig. 16). These results are consistent with those obtained in studies with other Hsp90-dependent protein kinases (*118, 168*).

Effects of Hsp90 inhibition on the association of Cyclin A with Cdk2- Other Hsp90 dependent protein kinases become structurally and functionally unstable in the presence of geldanamycin (*106-108*). Since our results suggested that Cdk2 is a client of the Hsp90/Cdc37 chaperone complex, we hypothesized that inhibition of Hsp90's activity with geldanamycin would affect Cdk2's ability to associate with Cyclin A. This

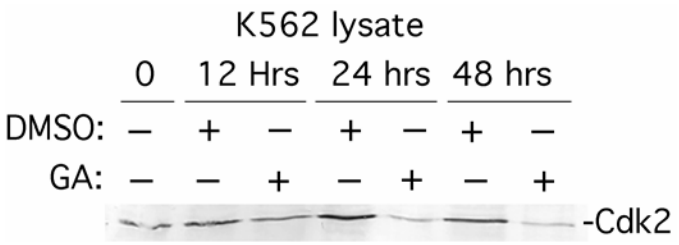


Fig. 16. **Endogenous Cdk2 protein expression is reduced in geldanamycin treated K562 cells.** K562 cells were grown in the presence of either DMSO or 0.1 μM GA for 0, 12, 24, or 48 hours. Samples were washed, lysed and quantitated by BCA assay. Lysates were then ran on SDS-PAGE and western blotted for Cdk2.

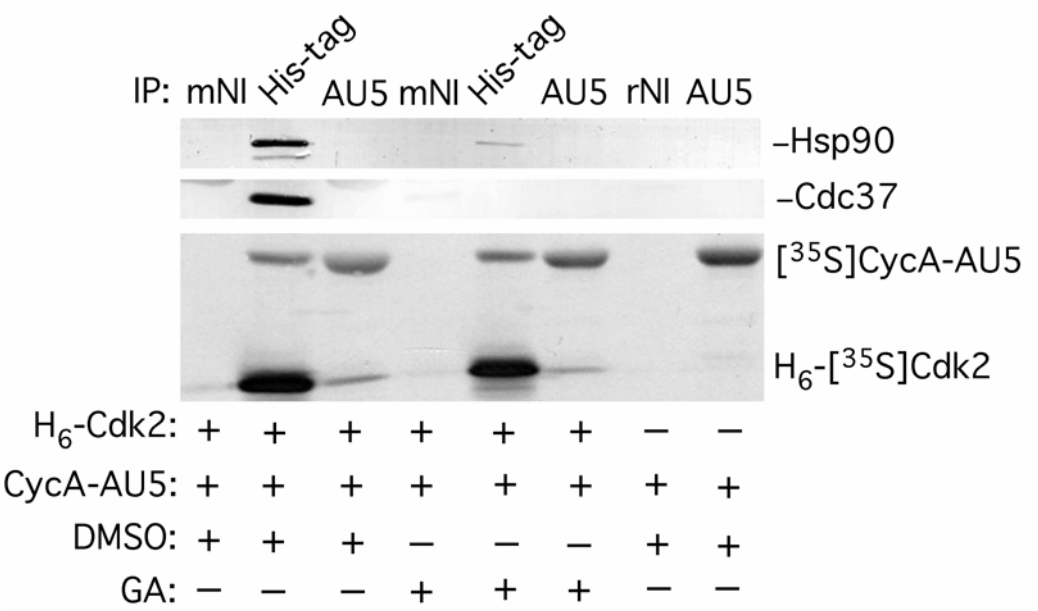


Fig. 17. **Cdk2 association with Cyclin A in the presence and absence of geldanamycin.** His-tagged Cdk2 and Cyclin A-AU5 were synthesized in the presence of either DMSO or 10 μg/ml GA for 30 min at 30 ^{0}C. Samples were mixed accordingly, and immunoabsorbed with the appropriate immunoresin. All samples were washed with P50T once, P500T twice, and P50T once more. Samples were then separated by SDS-PAGE and analyzed by autoradiography and western blotted for endogenous Hsp90 and Cdc37.

was also suggested by the work of Gerber *et al* which showed that Cdc37 defective yeast are unable to properly assemble Cdc28/Cln2 complexes (*100*), the equivalent of the Cdk2/Cyclin complex. We therefore predicted that the inability of Hsp90 to actively fold Cdk2 would prevent Cdk2 from attaining a stable conformation that could be recognized by Cyclin A. To test this hypothesis, we synthesized [^{35}S]-labeled His-tagged Cdk2 and Cyclin A-AU5 in separate TnT reactions in the presence or absence of geldanamycin. The reaction mixtures were then combined and incubated, and complexes immunoadsorbed accordingly. Samples were then analyzed by SDS-PAGE, autoradiography and western blotting for co-adsorption of endogenous Hsp90 and Cdc37 (Fig. 17).

Contrary to our prediction, the autoradiogram indicated that inhibition of Hsp90 function with geldanamycin had no significant effect on the stoichiometry of the interaction between Cyclin A with Cdk2. Band intensities indicated that when Cdk2 was immunoprecipitated Cyclin A co-adsorbed at a constant ratio in the presence or absence of geldanamycin. The reciprocal immunoprecipitation of Cyclin A indicated the same. Thus, currently in our hands the inhibition of Hsp90 with GA does not seem to have an effect on preventing Cdk2 from complexing with Cyclin A. This observation is consistent with previous findings in which it was shown that Cdc37 was unable to act as an assembly factor for Cdc28/Cln2 complexes (*169*). Nevertheless, the question of whether molecular chaperones play a role in cyclin and cyclin-dependent kinase associations deserves more rigorous attention.

Hsp90 and Cdc37 interactions with C-terminal domain deletion constructs of Cdk2- In our previous work, we observed that Hsp90 was able to interact with deletion

constructs of Lck that contained the last two strands of β–sheet (e.g., the β4/5-strands) in the N-terminal lobe and the entire C-terminal lobe of the catalytic domain of the kinase. In contrast Cdc37 was only found to stably interact with Lck deletion constructs that contained the N-terminal lobe αC-helix plus its β4/5-strands, in conjunction with the C-terminal kinase lobe (*163*). These findings were at variance to results from a study utilizing Cdk4 in which the G-box was found to be a critical motif required for Cdc37 binding (*141*). Therefore, we decided to use this same approach to define motifs required to trigger high-affinity binding interactions between Hsp90, Cdc37 and Cdk2.

Utilizing the crystal structure of Cdk2 (*129*), a series of C-terminal lobe deletion constructs of His-tagged Cdk2 were prepared (Fig. 18A). (This data is summarized in Fig. 19 and depicted in Fig. 20.) Each of these constructs was expressed by TnT in reticulocyte lysate, and samples were immunoadsorbed with anti-His-tag antibody in the presence or absence of molybdate. Samples were then analyzed via SDS-PAGE, western blotting, and autoradiography. Hsp90 and Cdc37 were co-adsorbed with the full length His-tagged Cdk2, which was used as a reference and positive control (Fig. 18A). Based on western blot intensities, the amount of the Hsp90 that was co-adsorbed with either the NL-β7 construct [which contains conserved kinase motifs I (the G-box) through VIB (the equivalent of β–sheets 6 and 7 of PKA (*123*)] or the NL-Cata construct [which contains motifs I through VI (the loop containing the catalytic Asn residue)] was approximately one quarter of that which was co-adsorbed with full length Cdk2 in the absence of molybdate. However, in the presence of molybdate, the amount of Hsp90 that co-adsorbed with the NL-β7 and the NL-Cata constructs was approximately 50% of the quantity of Hsp90 that co-adsorbed with the full-length protein. Furthermore, only

marginally detectable levels of Cdc37 were observed to co-adsorb with the NL-β7 and NL-Cata constructs, whether or not molybdate was present during the immunoabsorption. It is noteworthy that the amount of Hsp90 and Cdc37 that co-adsorbed with the NL-β7 and the NL-Cata Cdk2 constructs was far less than what was expected. Based on similar experiments carried out with deletion constructs of Lck and Cdk4 (*141, 163*), we predicted that these construct would bind Hsp90 and Cdc37 at 50-75% of the level bound by full length Cdk2 in the absence of molybdate, and at a level nearly equivalent to full length Cdk2 in the presence of molybdate.

The interaction of Hsp90 and Cdc37 with two other Cdk2 C-terminal domain deletion mutants [the NL-αE construct, which contains the αE-helix of the CL, but not the helix capping motif; and the NL construct which lacks the entire CL and terminates after β-sheet 5] was also different than expected from our previous results. In the absence of molybdate, no Hsp90 or Cdc37 that was detectable by western blotting, co-absorbed with these constructs (Fig. 18A). In the presence of molybdate, Hsp90 co-absorbed with the constructs and was detected by western blotting, whereas no co-adsorbing Cdc37 was detected (Fig. 18A). Based on our previous results (*163*), the NL and NL-αE constructs were predicted to interact weakly with Hsp90 in the absence of molybdate, and molybdate was predicted to stabilize the interaction of both Hsp90 and Cdc37 with the Cdk2 NL-αE construct.

While some of the result described above were somewhat different than expected, the data indicated that Hsp90, and to a much lesser extent Cdc37, was only able to bind Cdk2 constructs that contain N-terminal kinase lobe and C-lobe motifs through the αE-helix, complete with its capping motif. These results support our previous findings with

Lck (*163*), and again emphasize the importance of the stability of the αE-helix, and most likely its interface with the hinge-loop motif between the αC-helix and β4-strand in the N-terminal kinase lobe. However, in the presence of molybdate, Hsp90 was observed to bind the NL-αE and NL Cdk2 constructs that were void of stably bound Cdc37. This finding differs distinctly from previous work on Cdk4 (*141*), and our work with Lck (*163*), which demonstrated that Cdc37 was able to bind kinase constructs that contained a complete N-terminal lobe, especially after the addition of molybdate (*108, 170*).

Hsp90 and Cdc37 interactions with N-terminal domain deletion constructs of Cdk2- To similarly define the high affinity binding interactions of Hsp90 and Cdc37 with the N-terminal lobe of Cdk2, we prepared a series of N-terminal deletion constructs and tested them accordingly (Fig. 18B). (This data is summarized in Fig. 19 and depicted in Fig. 20.) Hsp90 was co-adsorbed, albeit at a very low level with theβ3-CL [which is missing kinase motif I (the G-box)], and marginally with the αC-CL [which starts at conserved motif III (the αC-helix which contains an invariant catalytic Glu residue)] Cdk2 constructs, with slight increases when molybdate was present to stabilize binding. Conversely, the β4-CL construct [which initiates after the hinge-loop in kinase motif IV] did not bring down any Hsp90, even in the presence of molybdate. These results are rather consistent with those obtained with N-terminal deletion constructs of Lck, in which the interaction of Hsp90 with N-terminal deletion constructs was lost upon deletion of the αC-helix (*163*). However, the interaction of Hsp90 with the β4-CL construct of Lck was stabilized, although at a low level, upon molybdate

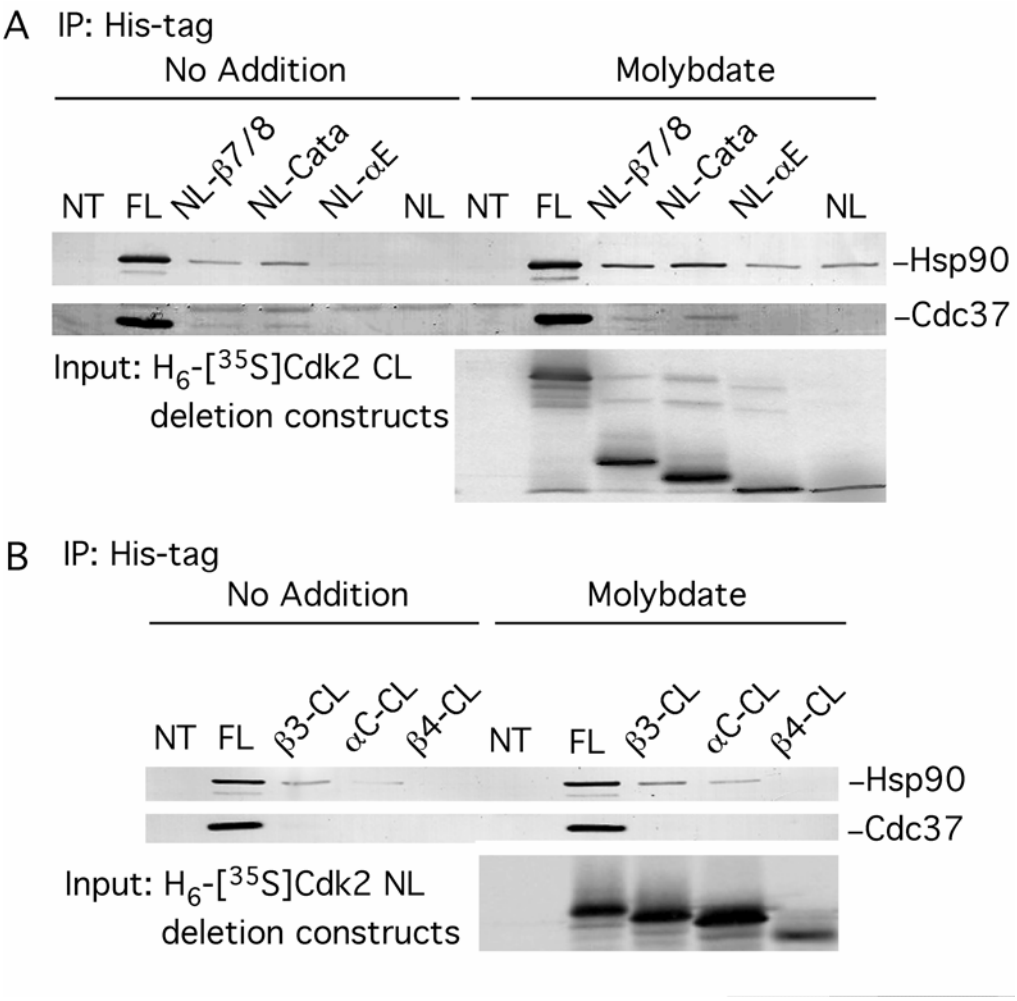


Fig. 18. **Interactions of Hsp90 and Cdc37 with His-tagged Cdk2 deletion constructs**. Using a no DNA template as a control for non-specific binding, His-tagged Cdk2 constructs were synthesized and labeled with ^{35}S-Met in reticulocyte lysate for 30 min at 30 ^{0}C, followed by immunoabsorption with anti-H_5 coupled immunoresin in the presence or absence of 17 mM molybdate. All samples were washed with P50T once, P500T twice, and P50T once more, no molybdate was present in the wash buffers. Next, samples were separated on an 8% SDS-PAGE gel and western blotted for endogenous Hsp90 and Cdc37. Input controls were separated on an 10% SDS-PAGE gel and analyzed by autoradiography. **(A)** His-tagged Cdk2 C-terminal deletion constructs. **(B)** His-tagged Cdk2 N-terminal deletion constructs. Each deletion construct and its interactions with Hsp90 and Cdc37 are further described in **Fig. 19**.

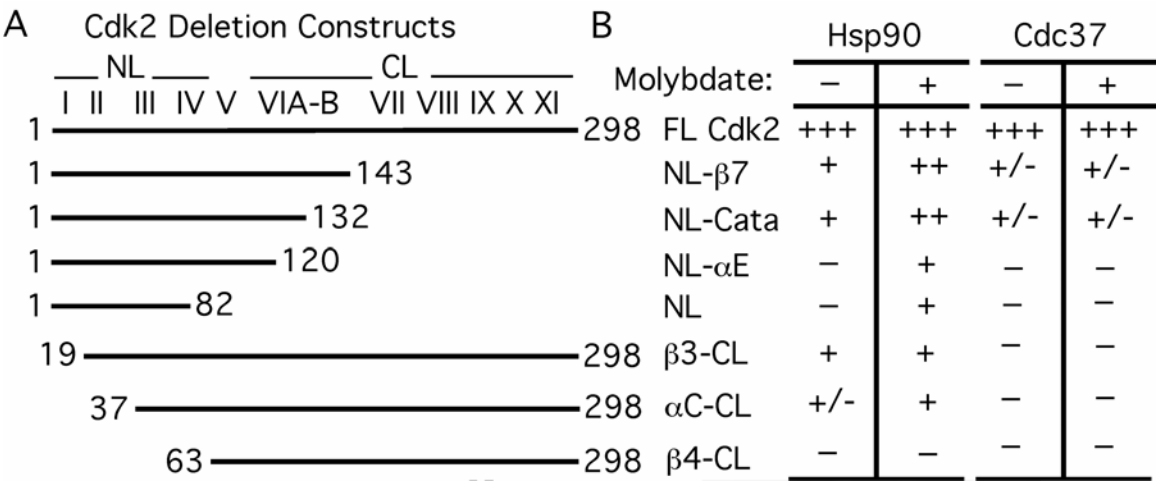


Fig. 19. **Summary of the His-tagged Cdk2 deletion constructs used for the analysis of Hsp90 and Cdc37 interactions. (A)** The figure on the left shows the placement of the NL and CL of Cdk2 above the conserved structural motifs designated by the Roman numerals (as proposed by Hanks and Hunter (*123*)). The numbers flanking each line indicate the first and last residues, respectively in that construct. **(B)** The chart on the right is aligned with the constructs to indicate the degree of Hsp90 and Cdc37 binding in the absence and presence of molybdate: +++, 100% of full length Cdk2; ++, less than 50% of full length; +, less than 25% of full length; +/-, marginal detection, less than 5% of full length; and -, no detectable binding.

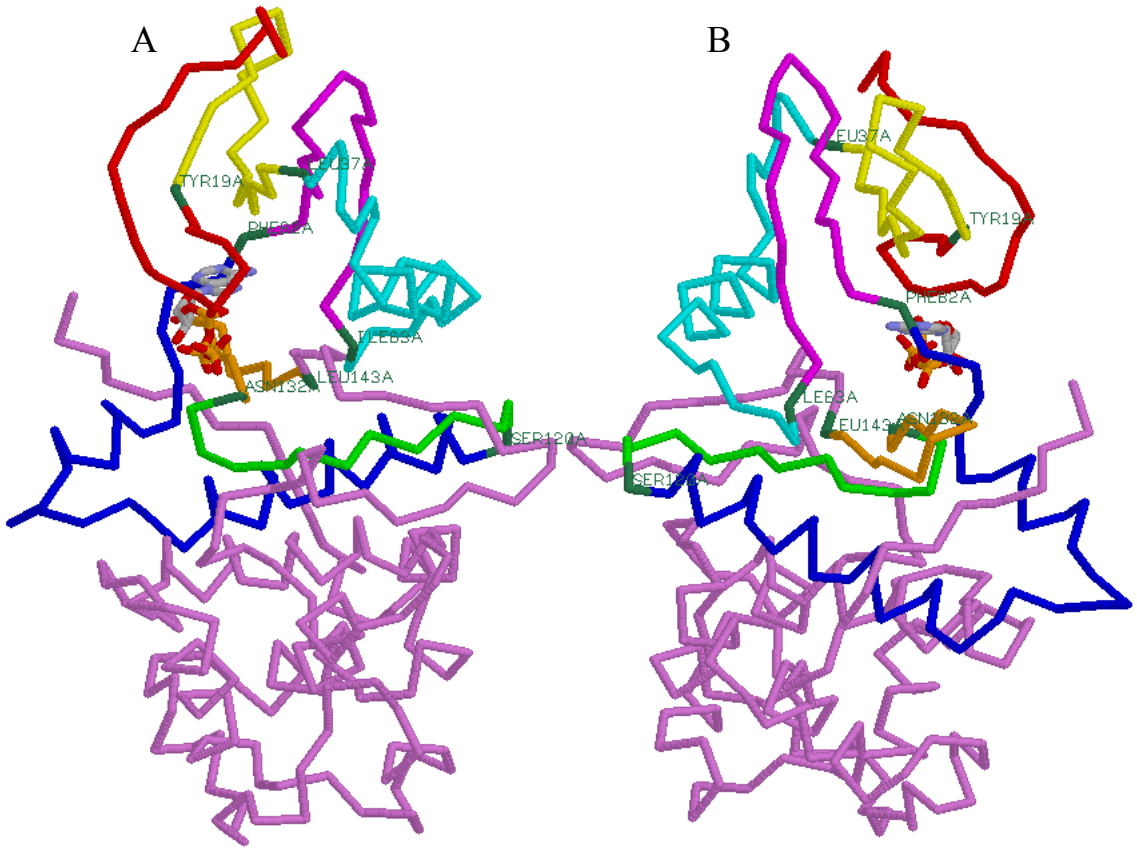


Fig. 20. **Depiction of the Cdk2 deletion mutant constructs.** A different color is shown for each segment of Cdk2 deleted in respect to **Fig. 19**: residues 1-19, red; residues 20-37, yellow; residues 38-63, cyan; residues 64-82, magenta; residues 83-120, blue; residues 121-132, green; residues 133-143, orange; residues144-298, violet. The kinase domain is shown with its ATP binding cleft facing either **(A)** toward or **(B)** away from the viewer (*129*).

addition, no doubt underlying the significance of the αC-helix and the hinge-loop motif.

Contrary to results obtained with N-terminal deletion mutants of Lck (*163*), no Cdc37 was detected co-absorbing with any of the N-terminal deletion constructs of Cdk2, even in the presence of molybdate. This result, however, was consistent with results obtained with Cdk4 (*141*), where the G-box was identified as a critical motif for Cdc37 binding. In contrast, Hsp90 and Cdc37 do co-absorb with the β3-CL construct of Lck (*163*), even in the absence of molybdate.

Discussion

Cdk2 is a cyclin-dependent kinase and a close relative of Cdk4, a well documented client of the Hsp90/Cdc37 chaperone complex (*125*). Moreover, previous studies have shown that Cdc37 associates with and is required for the proper function of Cdc28, the yeast homolog of Cdk2 (*100, 126, 169*). However, in other studies, a physical interaction of Hsp90 and Cdk2 has not been observed (*125*). Here we demonstrate that Cdk2 indeed physically interacts with both Hsp90 and Cdc37 and demonstrates classic client behavior upon inhibition of Hsp90 by GA.

The reduced rate at which Cdk2 turns over in K562 cells in the presence of GA indicates that Cdk2 may not require Hsp90 for maintenance of the stability of mature Cdk2 molecules. The kinetics Cdk2 turnover was much slower than that observed for ErbB2 (*171*), Raf (*172*) and oncogenic mutants of Lck and Hck (*146, 173*), which turn over at a much higher rate upon treatment with geldanamycin. Hsp90 is required both for

the proper folding of these kinases when they are newly synthesized, and for maintaining the stability of the kinases after they have matured to an active form.

However, our result is consistent with studies on the Hsp90-dependence of other protein kinases. The turnover of wild type Lck (*118, 173*), Hck (*146*) and Fyn (*168*) in cells is slow because mature kinase molecules do not depend upon Hsp90 for their stability. Geldanamycin-induced turnover of the kinases is primarily due to the inability of cells to replace mature kinase molecules, as they turn over at their normal rate, with newly synthesized kinase molecules, which are Hsp90-dependent and very labile in the presence of geldanamycin. Thus, the absence of a requirement for Hsp90 and Cdc37 to maintain the stability of mature Cdk2 molecules may explain why it is difficult to co-absorb Hsp90 and Cdc37 with Cdk2 from cultured cell lysates (data not shown). The Hsp90/Cdc37 dependent population of Cdk2 molecules would be limited to a small population of newly synthesized Cdk2 molecules, with the vast majority of Cdk2 in cultured cells being mature and stable. In contrast, the majority of Cdk2 molecules in TnT reticulocyte lysate are newly synthesized nascent polypeptides.

The results reported here also reaffirm the importance of the αC-helix in protein kinases for their proper recognition by and interaction with Hsp90 and Cdc37. The sc-53 anti-PSTAIRE antibody, which is directed against residues in the αC-helix of Cdk2, blocked the interaction of Hsp90 and Cdc37 with Cdk2. This observation explains results from earlier studies utilizing this antibody, which concluded that Cdk2 did not physically associate with Hsp90 and Cdc37 (*125*). This finding supports previous work with Lck and Cdk4, which indicated that the presence of the αC-helix was required for the interaction of Cdc37 with kinase molecules (*141, 163*). However, it can not yet be

concluded that the αC-helix is the site at which Cdc37 recognizes and binds kinase, as the binding of the antibody could block the interaction of Cdc37 by: (i) sterically inhibiting the interaction of Cdk2 with Cdc37; (ii) distorting the αC-helix, as antibodies are known to bind peptide epitopes in an extended conformation, (iii) altering the structure of other regions of the kinase that are dependent on the helix for folding or maintaining their structure; or (iv) a combination of these effects.

Currently, we favor the notion that the binding of the sc-53 antibody to the αC-helix globally distorts the N- teminal lobe and subsequently reduces the ability of Cdc37 to interact. This hypothesis predicts that the αC-helix is required to maintain a number of intra-molecular interactions that stabilize the protein and that distortion of the Cdk2 N-lobe by any number of mechanisms could lead to the loss of Hsp90 and Cdc37 interaction. We favor this notion, as it helps to explain the variant results obtain in studies on the Lck and Cdk4 kinases (*141, 163*).

While results from the deletion analysis of Cdk2 conflicted with some aspects of previous findings carried out on Cdk4 and Lck (*141, 163*), the primary points of each of these studies were upheld. The importance of the G-box in the Cdk4 study, as well as the stabilization of the αE-helix reported in the Lck study, were both shown to be critical for interaction of Hsp90 and Cdc37 with Cdk2. Nevertheless, the difference in the interaction of Cdc37 with C-terminal deletion mutants of Cdk4 and Cdk2 suggests that there are subtle structural differences between the protein kinases. Sequence alignment of the N-terminus of the two catalytic kinase lobes shows that Cdk4 contains an elongated αC-helix and extra amino acids within the β3-αC loop and the β4-β5 loop. The amino acid residues within these expanded sequences may provide a larger interface

for Cdc37 recognition and binding and/or added stability to the interaction of Cdc37 with Cdk4. On the other hand, the β3-αC loop of Cdk4 which is composed of seven continuous glycines may instead provide the increased flexibility that allows the N-teminal lobe to adopt the conformation that can stably interact with Cdc37.

Interestingly, the importance of the G-box for the interaction of both Cdk4 and Cdk2 with Cdc37 may also be related by the fact that the kinase activity of each protein can be inhibited by the phosphorylation of residues within this motif (*174*). This inhibition of kinase activity is most likely due to the distortion of the G-box conformation and may signify that slight perturbations of this motif could again have global effects upon the overall structure of the NL. What's more, it should be noted that the G-box of the client kinase c-Raf, which much like Cdk4, is critical for Cdc37 interaction (*141*) may also contain a possible regulatory phosphorylation site at S357 or S359. Furthermore, mutation of the third G residue in the G-box of B-Raf to Ala leads to the hyperactivation of its kinase activity, an observation that further supports the notion that slight changes in the conformation of this region can have significant effects on the structure of the NL (*175*).

The data presented here further support our previous findings that the stability of the interaction of Hsp90 with the catalytic domain of protein kinases requires structural motifs present in both lobes of the catalytic domain. As observed previously for Lck (*163*), stabilization of the C-terminal lobe's αE-helix, by extending the sequence to provide a proper capping motif, seems to be critical for maintaining Hsp90 and possibly Cdc37 interaction with Cdk2. The deletion construct containing a capped αE-helix is more likely to retain its α–helical structure and possibly the orientation of its side chains.

We postulate that this stabilized α-helix is then able to give the αC-β4 hinge-loop a complementary surface interface with which to interact, allowing in turn the semi-stable kinase construct to attain a conformation that is recognized by Hp90 and Cdc37. The Cdk4 C-terminal deletion studies also showed that the αC-β4 hinge-loop is required for Cdc37 binding, although interestingly enough, further stabilization of the hinge-loop structure by the structural motifs following it was not required (*141*). It is possible that this result is a consequence of expressing the Cdk4 N-terninal lobe as a C-terminal fusion to glutathione-S-transferase, in contrast to the expression of the isolated N-terminal lobe of Cdk2 described here.

Probably the most telling evidence supporting the significance of the hinge-loop structure is the work of Xu and co-workers comparing the ErbB1 and ErbB2 receptor tyrosine kinases (*176*). ErbB2 was demonstrated to require ongoing interactions with Hsp90 and Cdc37 to maintain its cellular stability, whereas ErbB1 required Hsp90 and Cdc37 only for its initial folding. The differences in the stability of each kinase were localized to residues in the hinge-loop region. However, our results support the notion that other structural motifs are also likely to be important for Hsp90 binding, such as the linker region, which interacts with the hinge-loop region and connects the two kinase lobes. Thus, it appears that while protein kinases share common structural features that are recognized by Hsp90 and Cdc37, it is now apparent that there are subtle differences in how a client kinase interacts with the Hsp90 chaperone complex, just as how there are differences in how kinase activities are regulated through alterations in the interactions between the two lobes of their catalytic domains.

CHAPTER IV

Induced Interactions of Hsp90 and Cdc37 with JNK1α1

Introduction

Hsp90 is a molecular chaperone that is essential for the biogenesis and maintenance of various signal transduction proteins that control all facets of eukaryotic cell physiology. Protein kinases, the largest and most intensely studied family of signal transduction proteins, often depend on Hsp90 for their cellular and functional stability. The human genome contains 518 genes (*120*) that encode protein kinases that when expressed form multiple cascading networks capable of exponentially amplifying a single signal.

Due to their diverse roles in controlling such processes as cell division, differentiation, and apoptosis these protein kinase signaling pathways must be regulated by opposing signaling pathways which in turn, create an intricate system of checks and balances, pitting one signaling pathway against several others in order to determine the fate of the cell. While it is understood that not all protein kinases are dependent on Hsp90, typically at least one member of a signaling pathway or of an opposing signaling pathway is an Hsp90 client, thus suggesting Hsp90 also plays a major role in indirectly regulating numerous signal transduction events.

Hsp90 and its "kinase specific co-chaperone" Cdc37 physically interact with protein kinases to support the proper folding and/or stabilization of their dynamic structure (reviewed in (*96, 97*). While this interaction has been proposed to be initiated by Cdc37 recruitment of the kinase client to the Hsp90 chaperone machine (*106, 108,*

111, 125, 144, 145), interaction of Hsp90 with client kinases has been observed in the absence of bound Cdc37 (*177*). Nonetheless, client kinases are thought to be processed by Hsp90 and Cdc37 by a mechanism that is dependent on Hsp90's nucleotide-mediated conformational switching (*106-108, 110, 163*).

Specific structural motifs within the bi-lobal structure of protein kinases are required for productive Hsp90 and Cdc37 chaperone interaction (*108, 141, 163*). While these motifs are located primarily in the N-terminal lobe (NL) of the kinase's catalytic domain, motifs present in the C-terminal lobe (CL) also contribute to the interaction of Hsp90 and Cdc37 with kinase domains. However, the exact manner by which kinase motifs are recognized by Hsp90 and Cdc37, and how the Hsp90/Cdc37 interactions with these motifs help facilitate the folding of the kinase is not known. Also not understood is why are some protein kinases clients of Hsp90 and Cdc37 while others are not, and which chaperone is responsible for this selection, Hsp90 or Cdc37?

Mitogen and stress activated protein kinase (MAPK/SAPK) pathways are conserved networks of protein kinases arranged in multiple tiers capable of transducing numerous signals. At the distal ends of the signaling pathway are the MAPK/SAPK themselves, p38, ERK, and JNK, which phosphorylate specific sets of proteins, most often transcription factors which result in altered gene expression. Initiating and/or transducing the signaling cascade are the upstream kinases, MAPK kinases (MAPKK) and MAPKK kinases (MAPKKK), which phosphorylate and activate the downstream effector MAPK/SAPKs (*130, 178*).

Most MAPK/SAPKs are comparatively similar in structure and function and have not been shown to be Hsp90 or Cdc37 clients. However, many of the upstream kinases

(MAPKKs and MAPKKKs) that activate MAP kinases are Hsp90 clients (*179-182*). The structures of the majority of MAPK/SAPKs are relatively simple when compared to the upstream MAPKKs and MAPKKKs Hsp90 client kinases, which contain one or more additional regulatory domains responsible for the binding of specific effectors. While many MAPK/SAPKs contain additional motifs either upstream or downstream of their catalytic core (*131, 183, 184*), these kinases lack major regulatory domains, and as a result, the activation of MAPK/SAPKs is usually stimulated simply by the phosphorylation of specific sites in their activation loop (*185*).

However, most MAPK/SAPKs possess two non-catalytic structural motifs (NCSMs) flanking the kinase domain that are currently not found in Hsp90 client kinases. The N-terminal non-catalytic structural motif (NSM) is two anti-parallel β-sheets preceding motif I (G-box) which lie on top of the other β-sheets of the NL and form a β-sandwich. The C-terminal non-catalytic structural motif (CSM) is a large extended region that is not completely defined in MAPK/SAPK crystal structures. Although the regions that are defined indicate that the CSM wraps around the kinase domain interacting with both the alpha-E helix in the CL and αC-helix in the NL. These MAPK/SAPK structural motifs have been shown to be important in stabilizing kinase activity and in the cellular localization of the kinase, as well as the binding of substrates, upstream kinases, scaffold proteins and phosphatases (*186-189*). However, whether these motifs provide MAPK/SAPKs with the added structural stability that allows them to function independent of Hsp90 and Cdc37 has not been determined.

In this report, we have used deletion mutagenesis and the classic Hsp90 inhibitors, geldanamycin (GA) and molybdate, to examine the role that the N- and C-terminal

flanking motifs play in mediating Hsp90 and Cdc37 interactions with the SAPK, JNK1α1. Consistent with our previous findings, we show that Hsp90 and Cdc37 recognize structural features found within the N-terminal lobe of the protein kinase, while Hsp90 also recognizes other features within the C-terminal lobe (*108, 163*). Additionally, our results suggest a novel mechanism by which Cdc37 recognizes kinases and possibly recruits them to Hsp90. Consequently, these results increase our understanding of why some protein kinases interact with Hsp90 and Cdc37, and some apparently do not.

Experimental Procedures

Plasmids. The original pcDNA 3.1-Flag-JNK1α1 plasmid was a gift from Dr. Roger Davis. All plasmids coded for an N-terminal Flag-tagged JNK construct under a T7 promoter. The N-terminal truncation construct, JNKΔNt (residues 28-384), was made by amplifying the original pcDNA 3.1-Flag-JNK1α1 by PCR, but using a 5' primer that deleted the first 27 residues. The 5' primer also contained 15 bases on its 5' end that were the same as another primer that encoded the Flag-tag. The first PCR product was amplified again using this Flag-tag primer and then ligated into pcDNA3.1-Topo (Invitrogen) according the manufactures specifications. The C-terminal truncation constructs, JNKΔCt (residues 1-322) and JNKΔNtCt (residues 28-322), were made by mutating the 323 codon into a stop codon via QuickChange (Stratagene). Mutation of the activation loop residues T183 and Y185 (TPY) to Ala and Phe (APF) or Glu (EPE) was also done using QuickChange.

Co-immunoprecipitations of chaperones with Flag-tagged JNK1α1 deletion constructs. Using a no DNA blank as a control for non-specific binding (NS), each construct was synthesized and radiolabeled with [^{35}S]-Met by coupled transcription/translation in 25-35 μl T7 Quick Master Mix (Promega) for 30 minutes at 30^0 C. In indicated experiments synthesis was done in the presence of either DMSO or 10 μg/ml GA, and in some experiments 20 mM molybdate was added after synthesis was completed. All immunoprecipitation samples were immediately placed on ice, clarified by centrifugation by a Fisher 235c microfuge at 4^0C for 5 min and immunoabsorbed with 25-30 μl of anti-mouse immunoresin coupled to mouse anti-Flag (Sigma) antibody. Immunoresins were then washed once with P50T (10 mM Pipes (pH 7.2), 50 mM NaCl, and 0.5% Tween-20), two times with P500T (same as P50T except with 500 mM NaCl), and again with P50T. No molybdate was present in the wash buffers. Finally, the samples were boiled in SDS sample buffer, separated on an 8% SDS-PAGE gel, transferred to PVDF membrane, analyzed by autoradiography for equal expression and blotted for both endogenous Hsp90 and Cdc37 (*108*).

JNK kinase assay. Immunoresins were washed twice with P500 (no Tween-20) and twice with P150. Samples were then incubated with 30 μl of kinase buffer (25 mM Pipes pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 10 mM glycerophosphate, 2.5 mM NaF, and 200 μM ATP) and 0.5 μg of c-Jun (1-89)-GST (Cell Signaling) for 15 minutes at 37^0C. Finally, samples were boiled in SDS sample buffer, separated on an 8% SDS-PAGE gel, transferred to PVDF membrane, and blotted with anti-phospho c-Jun (Ser63) (Cell Signaling Technology).

Results

JNK1α1 deletion constructs interact with Hsp90 and Cdc37. The c-Jun N-terminal kinase (JNK) group of kinases is encoded by three genes that produce a total of 10 splice variants. Each splice variant differs in its substrate binding motif and/or C-terminal structural motif size (*190*). In this work, we have studied the 46 kDa isoform, JNK1α1, which contains the smaller of the C-terminal structural motifs.

While the cellular stbility of numerous protein kinases have been shown to depend upon the Hsp90/Cdc37 molecular chaperone complex (*14, 15, 96, 97, 135, 191, 192*), the cellular stability of JNK does not seem to be affected by Hsp90 inhibition (*181, 193, 194*). Thus, JNKs appear to be Hsp90-independent kinases. To understand this phenomenon, we attempted to determine what structural motifs within JNKs confer or defer Hsp90 and Cdc37 interaction. Structural alignment and comparisons of the crystal structures of MAPK/SAPK members ERK (*184*), JNK (*131*) and p38 (*183*) with Hsp90 dependent kinases Cdk2 (*129*), Akt (*195*), and Lck (*196*) indicated that the MAPK/SAPK member kinases possess additional structural motifs at the immediate ends of both their N- and C-terminal lobes of their catalytic domains (alignment not shown). The N-terminal motif consists of two additional strands of anti-parallel β−sheet yielding a β-sandwich like structure. The extended C-terminal motif of JNK wraps around the NL to pack an α-helix against the αC-helix which contains a Glu required for catalysis of phosphate transfer. An α-helix present in cyclins similarly interacts with the αC-helix in Cdks to stabilize its rotation and direct the side chain of its catalytic Glu into the active site (*197*).

Additionally, the position of the activation loop was investigated to determine if it played some role in Hsp90 and Cdc37 interaction. This was suggested by the fact that the activation loops in the inactive conformation of MAPK/SAPKs lie close to the G-box, a motif found to be required for Cdc37 interaction with both Cdk4 and Cdk2 (*141*). However, when the activation loops of MAPK/SAPKs are phosphorylated they move away from the G-box and fold down along the CL which results in activation of the kinases (*185*).

Our previous work indicated that antibody directed against sequences within the αC-helix blocked the binding of Cdc37 to Cdk2, leading us to speculate that Cdc37 may play a role in stabilizing the rotation of the αC-helix within the NL of protein kinases. To test this hypothesis, we prepared a series of wild type N-terminal Flag-tagged deletion constructs of JNK1α1: ΔNt (28-384), ΔCt (1-322), and ΔNtCt (28-322). Furthermore, to test whether the position of the activation loop played some role in Hsp90 and Cdc37 interaction residues T183 and Y185 (TPY) were mutated to either Ala and Phe (APF) or both to Glu (EPE), in order, to code for a constitutively inactive or active kinase, respectively. [^{35}S]-labeled full length JNK, deletion constructs and point mutants were synthesized by coupled transcription/ translation in reticulocyte lysate, immunoprecipitated with anti-Flag agarose resin, and assayed for co-absorption of Hsp90 and Cdc37 by western blotting (Fig. 21). As previous work suggested (*181, 193, 194*), Hsp90 and Cdc37 did not co-absorb with full length JNK or any of its point mutants. However, Hsp90 co-absorbed with all of the deletion constructs. In contrast, Cdc37 co-absorbed with only the ΔNt construct and was only marginally detectable with the ΔNtCt construct. This reduced binding of Cdc37 to ΔNtCt constructs is possibly a result of the

added instability afforded by the removal of the CSM. Furthermore, it appears that mutating of T183 and Y185 had no significant effect on Hsp90 or Cdc37 interaction (Fig. 21).

These observations (Fig. 21) were somewhat contrary to our previous speculation that Cdc37 may play a role in stabilizing the rotation of the αC-helix within the NL of protein kinases (*108, 163*). We anticipated that the ΔCt construct would bind Cdc37 and Hsp90, while the ΔNt would have little or no interaction with Hsp90 or Cdc37. Instead, co-adsorption of Cdc37 with the ΔNt construct suggests that the NSM prevents Cdc37 from recognizing the motifs required for its binding. Thus, the role of Cdc37 may be to stabilize the β-sheet structure in the N-terminal lobe rather than the αC-helix. This notion is further supported by the significant reduction in the co-adsorption of Cdc37 with the ΔNtCt compared to the ΔNt construct, indicating that exposure of the backside of the αC-helix does not enhance the binding of Cdc37 to the mutant JNK construct.

The association of Cdc37 with all nascent client kinases that have been investigated so far has always been accompanied by Hsp90 (*108, 145, 163, 170*). Therefore, the co-absorption of Hsp90 with the ΔNt construct was not unexpected (Fig. 19). However, the co-absorption of Hsp90 and not Cdc37 with the ΔCt construct suggested that Hsp90 may interact with the ΔNt and ΔCt constructs differently. Thus, the interaction of Hsp90 with the ΔNt construct may be coordinated by Cdc37, while the interaction with the ΔCt construct may be due to the unmasking of another putative binding site that is recognized by Hsp90 only. This hypothesis is consistent with our previous findings that the interaction of Hsp90 with multiple discrete segments of client kinases is required to trigger the high affinity binding of Hsp90 (*108, 163*).

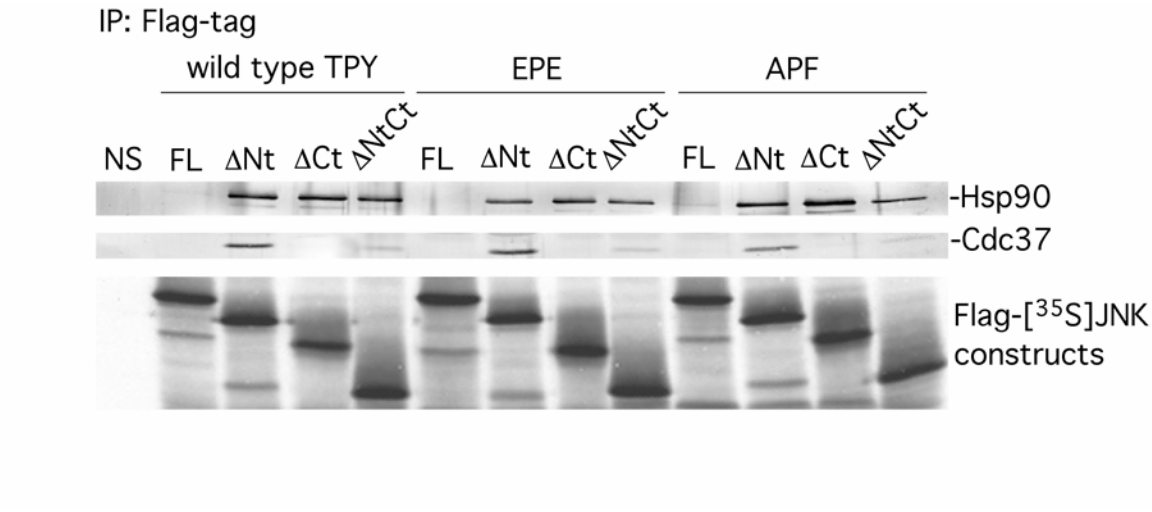


Fig. 21. **Interactions of Hsp90 and Cdc37 with JNK1α1 deletion constructs and activation loop point mutants.** Using a no DNA blank as a control for non-specific (NS) binding, Flag-tagged JNK1α1 deletion constructs and point mutants were synthesized and labeled with ^{35}S-Met in 35 μl of reticulocyte lysate for 30 min at 30 ^{0}C, followed by immunoabsorption with anti-Flag immunoresin. All samples were washed with P50T once, P500T twice, and P50T once more. Samples were then run on SDS-PAGE and western blotted for co-absorbing endogenous Hsp90 and Cdc37, according to "Experimental Procedures".

Geldanamycin effects on Hsp90 and Cdc37 interactions with JNK1α1 deletion constructs. To further characterize the interactions of Hsp90 and Cdc37 with the JNK deletion constructs, we tested the effects of the Hsp90 inhibitor geldanamycin on both the ability of the JNK constructs to bind Hsp90 and Cdc37 and to phosphorylate the N-terminus of c-Jun (Fig. 22). Geldanamycin works by binding to the N-terminal ATP binding site of Hsp90 (*37*), which blocks ATP-induced "clamping" of the N-terminal domains within dimeric Hs90, and prevents Hsp90 and Cdc37 from forming a high-affinity salt-resistant heterocomplexes with client kinases (*107*). This property of geldanamycin allows one to test whether a client kinase interacts with Hsp90 and Cdc37 in the classical manner by observing whether the chaperones dissociate upon washing with buffer containing 0.5M NaCl (*107, 108*).

Full length wild type JNK and deletion mutant constructs were synthesized and [^{35}S]-labeled in coupled transcription/ translation in reticulocyte lysate, and immunoabsorbed with anti-Flag agarose resin. After washing the resins with buffer containing high salt, the samples were analyzed for co-absorption of Hsp90 and Cdc37 by western blotting (Fig. 22). Similar to the results described above, in the absence of geldanamycin, Hsp90 was co-adsorbed with each of the deletion mutants, while Cdc37 co-absorbed with the ΔNt construct and marginally with the ΔNtCt construct. Interestingly, in the presence of geldanamycin, Hsp90 still co-absorbed with the ΔNt and ΔNtCt constructs, although to a much lesser degree than in the absence of geldanamycin. However, the amount of Hsp90 that co-adsorbed with ΔCt was significantly reduced in the presence of geldanamycin. In addition, no Cdc37 co-absorbed with any of the deletion constructs in the presence of geldanamycin. Furthermore, no deletion construct

was able to phosphorylate the N-terminus (Ser63) of c-Jun after being synthesized in the absence or presence of geldanamycin, whereas full length JNK was able to phosphorylate the N-terminus of c-Jun after being synthesized under either condition. Thus, the NCSMs are required to maintain kinase activity, and the binding of Hsp90 and/ or Cdc37 does not correct the functional defects induced upon deletion of these motifs from JNK. This supports previous findings which demonstrated that the NSM is required for kinase activity in Erk2 (*187*).

Again, these observations (Fig. 22) did not totally support the predictions of our original hypothesis that Hsp90 and Cdc37 would be completely dissociated from the mutant kinase constructs in the presence of geldanamycin. True to previous observations (*107, 108*), Cdc37 did dissociate from all of the constructs in the presence of geldanamycin, indicating that Cdc37 can not productively interact with client kinases when they are bound to Hsp90 in its geldanamycin-bound conformation. In addition, the notable reduction in the amount of Hsp90 that co-absorbed with the ΔCt construct in the presence of geldanamycin suggests that Hsp90 is being arrested in its open "unclamped" conformation. This observation suggests that Hsp90 interacts with the ΔCt construct in a manner similar to its previously characterized interactions with natural client kinases (*108, 135*). Thus, Hsp90 may recognize and interact with specific structural motifs on natural client kinases that are masked in MAPK/SAPKs by the CSM.

However, the finding that Hsp90 in its geldanamycin-bound conformation did not completely dissociate from the ΔNt and ΔNtCt constructs suggests that Hsp90 might be interacting with these constructs in a novel manner that has not been previously observed. This interaction was dependent on the removal of the NSM and not mediated by Cdc37,

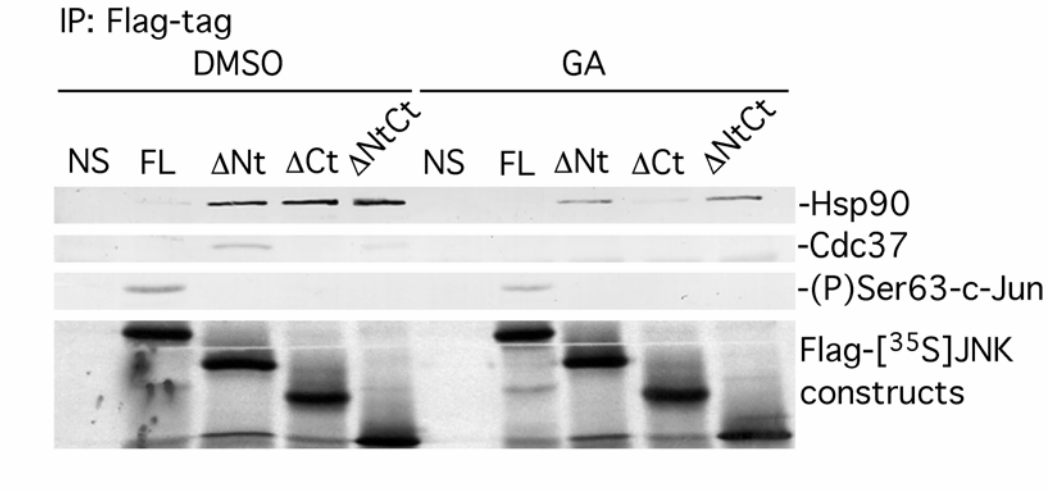


Fig. 22. **Kinase activity and interactions of Hsp90 and Cdc37 with JNK1α1 deletion constructs in the absence and presence of geldanamycin.** Using a no DNA blank as a control for non-specific (NS) binding, Flag-tagged wild type JNK1α1 deletion constructs were synthesized and labeled with ^{35}S-Met in 25 μl of reticulocyte lysate for 30 min at 30 ^{0}C in the presence of either DMSO or 10 μg/ml GA, followed by immunoabsorption with anti-Flag immunoresin. All samples were washed twice with P500 and twice more with P150. Kinase buffer and c-Jun (1-89)-GST was then added. Samples were then incubated at 37 ^{0}C for 15 min and then run on SDS-PAGE and western blotted for phospho c-Jun (Ser63) and co-absorbing endogenous Hsp90 and Cdc37, according to "Experimental Procedures".

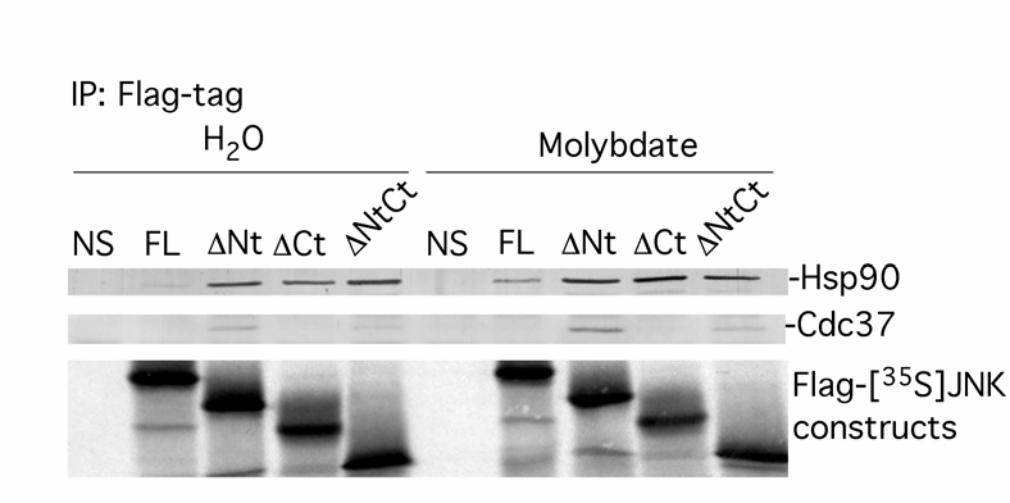


Fig. 23. **Interactions of Hsp90 and Cdc37 with JNK1α1 deletion constructs in the absence and presence of molybdate.** Using a no DNA blank as a control for non-specific (NS) binding, Flag-tagged wild type JNK1α1 deletion constructs were synthesized and labeled with ^{35}S-Met in 25 μl of reticulocyte lysate for 30 min at 30 ^{0}C followed by immunoabsorption with anti-Flag coupled immunoresin in the presence or absence of 17 mM molybdate. All samples were washed with P50T once, P500T twice, and P50T once more. Samples were then run on SDS-PAGE and western blotted for co-absorbing endogenous Hsp90 and Cdc37, according to "Experimental Procedures".

suggesting that Hsp90 may interact with client kinases through more than one mechanism.

Molybdate effects on Hsp90 and Cdc37 interaction with JNK1α1 deletion constructs. The addition of molybdate stabilizes interactions of Hsp90 and Cdc37 with client proteins by locking Hsp90 in its clamped "slow on/slow off" conformation (*107, 108, 163*). This allows Hsp90 and Cdc37 to form highly salt resistant heterocomplexes with the client protein. To further characterize the interactions of Hsp90 and Cdc37 with the wild type JNK deletion constructs, full length JNK and each deletion construct was synthesized by coupled transcription/ translation in reticulocyte lysate, and immunoprecipitated with anti-Flag agarose resin in the presence or absence of molybdate. The immuno-resins were then washed with buffer containing 0.5 M NaCl, and analyzed for co-absorbing Hsp90 and Cdc37 by western blotting (Fig. 23). In the presence of molybdate, the amount of Hsp90 that co-absorbed with each of the deletion constructs was equal to or greater than the amount co-adsorbed in the absence of molybdate. Cdc37 again co-absorbed with ΔNt with a subtle increase in the amount co-adsorbed in the presence of molybdate. However, the amount of Cdc37 that co-absorbed with ΔNtCt remained marginal. These observations are consistent with the effect of molybdate on the interactions of Hsp90 and Cdc37 with natural client kinases (*107, 108, 163*). Surprisingly, in the presence of molybdate, Hsp90 but not Cdc37 co-adsorbed with full length JNK.

Discussion

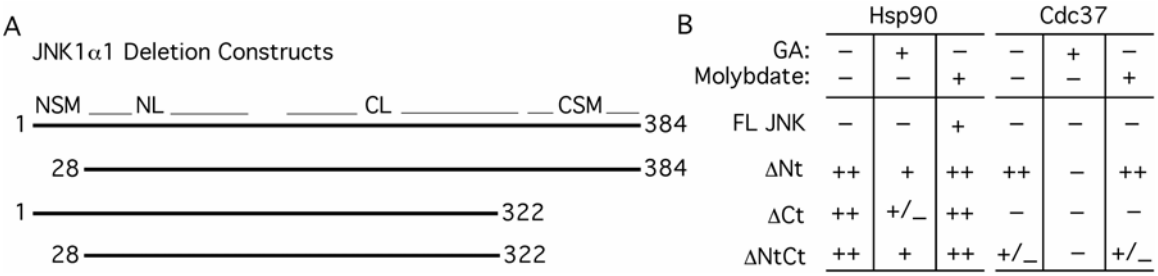
JNK1α1 is not an Hsp90 or Cdc37 associating client kinase. However, in this report we demonstrate that deletion of the non-catalytic structural motifs present at the N- and C-terminus of JNK1α1 can induce the binding of Hsp90 and/or Cdc37 (summarized in Fig. 24 and depicted in Fig. 25). The characteristics of these induced interactions only partially resemble those of Hsp90/Cdc37 with natural client kinases, which are weakened by geldanamycin while strengthened by molybdate (*107, 108, 163*). Furthermore, our findings suggest that Hsp90 may interact with protein kinases through two distinct sites. This hypothesis is supported by the differences observed between the binding of Hsp90 to the ΔNt and ΔCt constructs in the absence and presence of geldanamycin. Previously, deletion mutagenesis work with Lck also suggested that Hsp90 recognizes at least two distinct sites in kinases, as Hsp90 associates with non-overlapping constructs of both the NL and CL of Lck in the presence of molybdate (*108, 163*). Thus, these results suggest that Hsp90 and Cdc37 may recognize and bind specific structural motifs whose accessibility are masked or whose conformations are altered or stabilized by their interactions with the NSM and CSM in MAPK/SAPKs, and suggest a rationale for why some kinases interact with Hsp90 and Cdc37, and some do not.

The Hsp90 interaction site, which is "unmasked" by the deletion of the NSM, is recognized by Hsp90 even in the presence of geldanamycin, while the interaction of Cdc37 with this site remains sensitive to inhibition by geldanamycin. Notably, the characteristics of interaction of Hsp90 and Cdc37 with the ΔNt construct in the presence of GA is similar to their interactions with Akt (*177*). Akt appears to remain associated with Hsp90 after its maturation and dissociation of Cdc37. Crystallographic studies

indicate that the C-terminus and N terminus of the αB and αC-helices in Akt are intrinsically unstructured in the absence of the phosphorylation of S474 (*195*), which facilitates the activation of Akt (*198*). The congruence of this data suggests that the deletion of the NSM likely prevents the NL of JNK from folding properly, and that the stability of Hsp90's interaction with mis-folded or intrinsically disordered polypeptides is not dependent on nucleotide-mediated conformational switching.

The characteristics of the interaction of Hsp90 with the ΔCt construct in the presence and absence of geldanamycin is typical of the characteristics of its interactions with most Hsp90-dependent kinases (*108, 135*). This observation suggests that the site that is unmasked or whose conformation is altered upon deletion of the CSM contains a binding site for Hsp90 that modulates Hsp90's nucleotide-mediated conformational switching. However, the lack of interaction of Cdc37 with the ΔCt construct indicates that simply exposing the face of the αC-helix is not sufficient to induce Cdc37 binding, as we had previously speculated (*108, 163, 170*).

The interaction of Cdc37 with the ΔNt and ΔNtCt JNK constructs is consistent with previous work that indicated that Cdc37 interacts with motifs present in the NL of the catalytic domain of protein kinases (i.e., Lck, HRI, Cdk4, and Cdk2) (*108, 141, 170*). Our current findings further support the notion that the role of Cdc37 in chaperoning protein kinase folding is to stabilize the N-terminal lobe of the protein. However, our current work suggests that Cdc37 may mimic the role of the NSM β-sheet structure in stabilizing the structure of the NL of MAPK/SAPKs, a role distinctly different than our previous hypothesis that Cdc37 may interact directly with the αC-helix of kinases to stabilize its orientation within the catalytic site (*108, 163, 170*).



	Hsp90			Cdc37		
GA:	–	+	–	–	+	–
Molybdate:	–	–	+	–	–	+
FL JNK	–	–	+	–	–	–
ΔNt	++	+	++	++	–	++
ΔCt	++	+/–	++	–	–	–
ΔNtCt	++	+	++	+/–	–	+/–

Fig. 24. **Summary of the Flag-tagged JNK deletion constructs used for the analysis of Hsp90 and Cdc37 interactions. (A)** The figure on the left depicts the location of NSM, NL, CL and CSM of JNK1α1. The numbers flanking each line indicate the first and last residues, respectively of that construct. **(B)** The chart on the right is aligned with the constructs to indicate the degree of Hsp90 and Cdc37 binding in the absence and presence of GA and molybdate: ++, 100-90% of maximum binding; +, 50% of maximum binding; +/-, marginally detectable, less than 10% of maximum binding; -, no detectable binding.

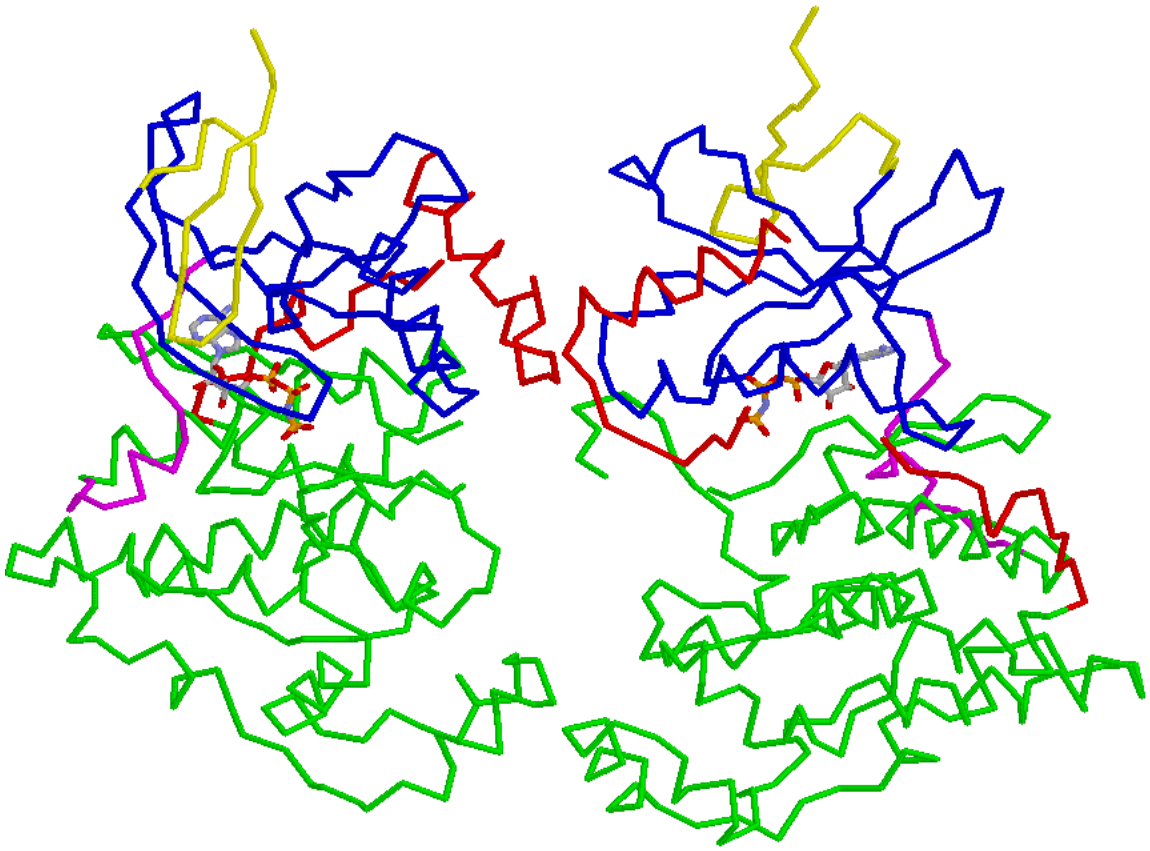


Fig. 25. **Depiction of the non-catalytic structural motifs studied.** Using the JNK3 crystal structure (*131*) (PDB: 1JNK) as a model the following structural features are depicted: NSM, yellow; NL, blue; linker, magenta; CL, green; CSM, red. ATP binding cleft is shown facing (A) toward, and (B) away from the viewer.

Our current findings suggest that Cdc37 may interact with client kinases to stabilize and/or nucleate the folding of the single-layered β–sheet structure present in the NL of Hsp90-dependent kinases. Single layers of β-sheets are not present in proteins, as they are unstable due to the exposure of the hydrophobic face of the sheet to water. Thus, the structure of the NL of kinases may require the interaction of its single layered β-sheet structure with Hsp90/Cdc37 to stabilize the NL until the synthesis of the kinase is complete, or the structure of kinase is adjusted to align or stabilize the interface between the two lobes. Regulation of the activity of protein kinases characteristically involves reorientation of the interface between the N- and C-lobes to properly align the amino acid residues that catalyze the phosphotransferase reaction (*162, 185, 199*).

Furthermore, at the interface between the NL and CL of protein kinases, the hydrophobic face of the NL provides the surface that interacts with the adenine ring of ATP. In the absence of bound nucleotide, the NL of protein kinases may be prone to denaturation, because of the exposure of the nucleotide-binding cavity to water. The notion that protein kinases may be unstable during the process of nucleotide exchange is supported by the observation that binding of the Src-family kinase inhibitor PP2 to a constitutively activated mutant of Lck, which requires Hsp90 and Cdc37 to maintain its function even after its initial maturation, stabilizes the protein in the presence of geldanamycin (*200*).

Cdc37 likely provides the major activity that stabilizes and/or facilitates the structure and/or folding of the NL of kinases. This hypothesis is supported by the observations that: (i) Cdc37 appears to interact exclusively with components of the NL of kinases; (ii) a deletion mutant of Cdc37 that does not bind Hsp90, binds kinase, and acts

as a dominant-negative inhibitor of kinase folding (*106, 145*); and (iii) the JNK ΔCt construct bound Hsp90, but not Cdc37. While Cdc37 may function to mimic the β-sheets of the NSM, it is also possible that deletion of the NSM simply allows the NL to adopt an altered conformation that is recognized by Cdc37. Thus, the NSM may not prevent Cdc37 interaction by simply physically blocking the binding site, but the NSM may stabilize the kinase structure so that it cannot be recognize by Cdc37. Furthermore, Cdc37 might possibly interact with the underside of the single layered β-sheet masking the hydrophobic residues that form the nucleotide binding cavity from the aqueous environment, prior to its packing against the CL.

While our current work suggests that the topology of the β-sheet structure of the NL of kinases is an important determinant for the interaction of a kinase with Cdc37, it does not address the disparities observed between the characteristics in Cdc37 binding to different protein kinase families. Deletion mutagenesis work with the Src-family member Lck showed that the first three strands of β-sheets within the NL were not needed for Cdc37 binding suggesting that the αC-helix and the fourth and fifth strands of β-sheets were the required structure for Cdc37 recognition (*163*), while work done with Cdk4 has indicated that the first three strands of β–sheet, the αC-helix and residues in the loop connecting the αC-helix to the β4-strand were required for Cdc37 binding (*141*). However, our work with Cdk2 essentially showed that nearly the complete NL was needed for Cdc37 binding. Taken together these observations suggest that subtle differences between the structures of the NL of kinases structure may equate to profound differences in the recognition and binding of Cdc37 to the kinase, which ultimately may only be understood through X-ray crystallography and NMR studies.

CHAPTER V

Discussion

Summary

The work presented in this thesis focuses the interactions between protein kinases and the molecular chaperones Hsp90 and Cdc37. Three different protein kinases from three different phylogenetic families were studied using deletion mutagenesis, in order to determine the structural motifs recognized and bound by Hsp90 and Cdc37. Each study utilized classic co-immunoadsorption in the presence or absence of the Hsp90 inhibitors GA and molybdate to verify Hsp90 and Cdc37 association. Although each study yielded different results, certain interactions remained consistent. These interactions being: (i) Cdc37 recognizes and binds primarily to the NL of protein kinases; (ii) this interaction is accompanied by Hsp90; and (iii) Hsp90 also recognizes certain structural features in the CL, that when present along with NL allow for nucleotide-mediated conformational switching of the chaperone complex.

The use of deletion mutagenesis to define the structural motifs required for Hsp90 and Cdc37 association is an effective technique for defining the broad strokes of the interaction. However, since these interactions do not seem to be based on amino acid sequence or a defined structure within the kinas,e using this technique creates a slippery slope when trying to determine the finer points of these interactions. This ambiguity is exemplified by the differences in Hsp90 and Cdc37 binding between the three different kinases. It is also illustrated by data that was not shown on the numerous Lck catalytic subunit construct (CAT) point mutants designed to disrupt chaperone interaction that

bound Hsp90 and Cdc37 with the same affinity as wild type, thus providing no information on what specific residues and motifs are critical for recognition and binding of Hsp90 and Cdc37 to protein kinases. Therefore, although this work yielded some critical information on the interactions of Hsp90 and Cdc37 with protein kinases, in the end it became akin to trying to split a hair with a sledgehammer. In retrospect, this is a likely outcome when using structural disrupting techniques to study a complex whose role is to bind and secure structurally unstable clients.

However, further work is needed to extend upon the findings in this report and explain some of the apparent discrepancies relative to kinase motifs recognized by Hsp90 and Cdc37. The study of a broader range of kinases from both Hsp90 dependent and independent families could provide more clues relative to what Hsp90 and Cdc37 recognize and bind. This should include kinases that function and/or exist as monomers or dimers and are either activated by hetero-phosphorylation or trans-autophosphorylation. Point mutagenesis of Cdk2 may also give some ideas of the specific residues and motifs needed for Hsp90 and Cdc37 binding. This approach may be productive with Cdk2 because its interaction with the chaperone heterocomplex does not seem to be as robust as Lck and may be easily disrupted by subtle sequence perturbations, as seen in the work with its relative Cdk4 (*141*). A finer dissection of the NCSMs of JNK1α1 is another approach that may yield more clues to what structural motifs are recognized and bound by Hsp90 and Cdc37, yet masked in MAPK/SAPKs. In the end though, the use of NMR and X-ray crystallography will be required to truly understand the interactions of protein kinases with Hsp90 and Cdc37.

Cdc37 Interactions

The role of Cdc37 is to recognize and bind specific structural motifs within the NL of protein kinases (*141, 163*). The KBD of Cdc37 is the most conserved domain across all species. Although its structure is unknown, the structural prediction program PONDR (*201*) suggests that most of the KBD is relatively unstructured. Initial NMR studies appear to confirm this prediction. It seems plausible that the unstructured nature of the KBD may be needed to adopt subtle variations in conformation, in order to bind the NL of different protein kinases. On the other hand, secondary structure prediction programs suggest that the first 40 residues of Cdc37, which are the most conserved phylogenetically, adopt a random coil or β–sheet structure that is followed by a coiled-coil motif, although the work with JNK1α1 currently suggests that the KBD structure somehow replaces a set of anti-parallel β-sheets that stabilizes the structure of the NL in native JNK.

Cdc37 is possibly the first chaperone to recognize and bind nascent protein kinase polypeptides as they emerge from the ribosome. The observation that Hsp90 and Cdc37 interact with nascent HRI polypeptides co-translationally first insinuated this possibility (*106*). Yet, the fact that Cdc37 only binds to the NL of kinases (*108, 163*), which often times is the first available domain for chaperone interaction, suggests that Cdc37, along with possibly the Hsp70 network, is the first chaperone to initiate the folding cycle of nascent kinases. The loss of Cdc37 association with the N-terminal deletion constructs of Lck and Cdk2 also support the notion that Cdc37 initiates proper kinase folding (*141, 163*). However, the fact that Hsp90 can still bind these N-terminal deletion constructs in

the presence of molybdate, suggests Hsp90 possibly binds after Cdc37 and at a different site (*108, 163*). These observations taken together suggest that proper binding and folding of wild type kinases may be accompanied by nucleotide mediated conformational switching of the chaperone complex independent of molybdate, and that Cdc37 may need to associate with the NL first, which is subsequently followed by the recruitment and binding of Hsp90.

The possibility that Cdc37 recruits the kinase polypeptide to Hsp90 puts forth a model in which Cdc37 acts similar, yet independent of Hop in transferring the kinase polypeptide from the Hsp70 network to the Hsp90 chaperone machine. Local amino acid sequence alignment of Cdc37 with Bag-1M and Hop has revealed that Cdc37 possesses a short conserved region with sequence similarity to each of these co-chaperones. The local sequence alignment of human Cdc37 and the Hsp70 nucleotide exchange factor, Bag-1M shows that Cdc37 possesses most of the highly conserved residues that are needed for Bag-1M to directly interact with the ATPase domain of Hsp70 (*75*). This interaction allows for the exchange of ATP for ADP along with the coordinated release of the client polypeptide. In Cdc37, this Bag-like motif (BLM) is predicted to have a coiled-coil structure and is found just outside of the highly conserved first 40 amino acids of the KBD [41-67 aa] (Fig. 26A). Bag has been shown to have a coiled-coil structure, and unpublished data of Jieya Shao has shown that Cdc37 does indeed interact with the ATPase domain of Hsp70.

The other local sequence alignment of human Hop and Cdc37 is found in both proteins on the N-terminal side of the Hsp90 binding domains: TPR2a in Hop [210-224 aa] and HBD in Cdc37 [131-146 aa] (Fig. 26B). This part of the HBD of Cdc37 has been

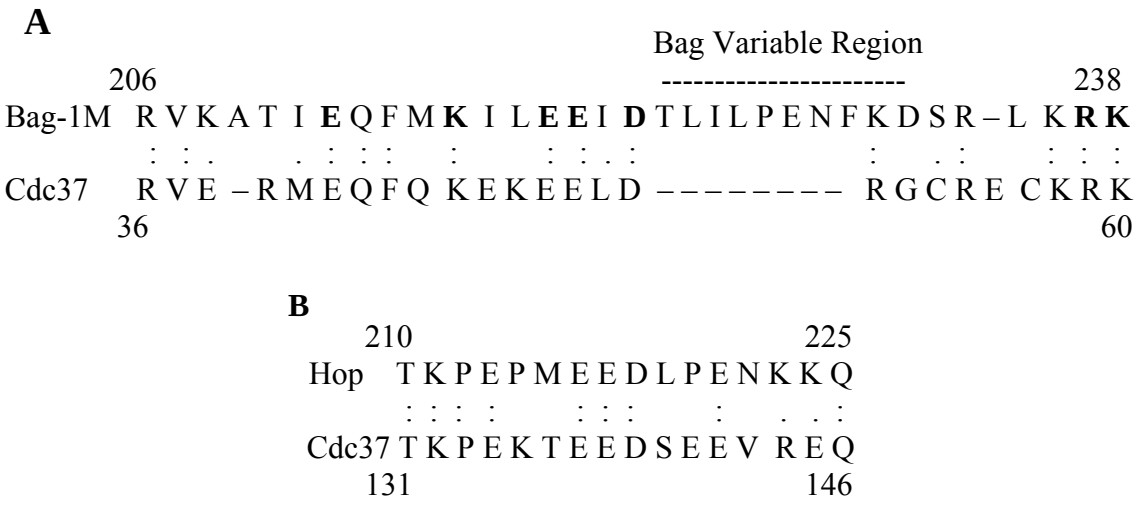


Fig. 26. **Local alignment of human Cdc37 with human Bag-1M and Hop. (A)** The helical region of Bag-1M that directly interacts with the ATPase domain of Hsp70 aligns with a region that lies within the KBD of Cdc37. The residues of Bag-1M that are conserved across all species and have shown to be required for Hsp70 interaction are shown in bold. Also, a variable region of Bag-1M and its homologues is indicated. (For further details see Shao, J., 2002 OSU Thesis pg.125) **(B)** The D2a domain of Cdc37 aligns with the region of Hop that precedes the Hsp90 binding TPR2a domain.

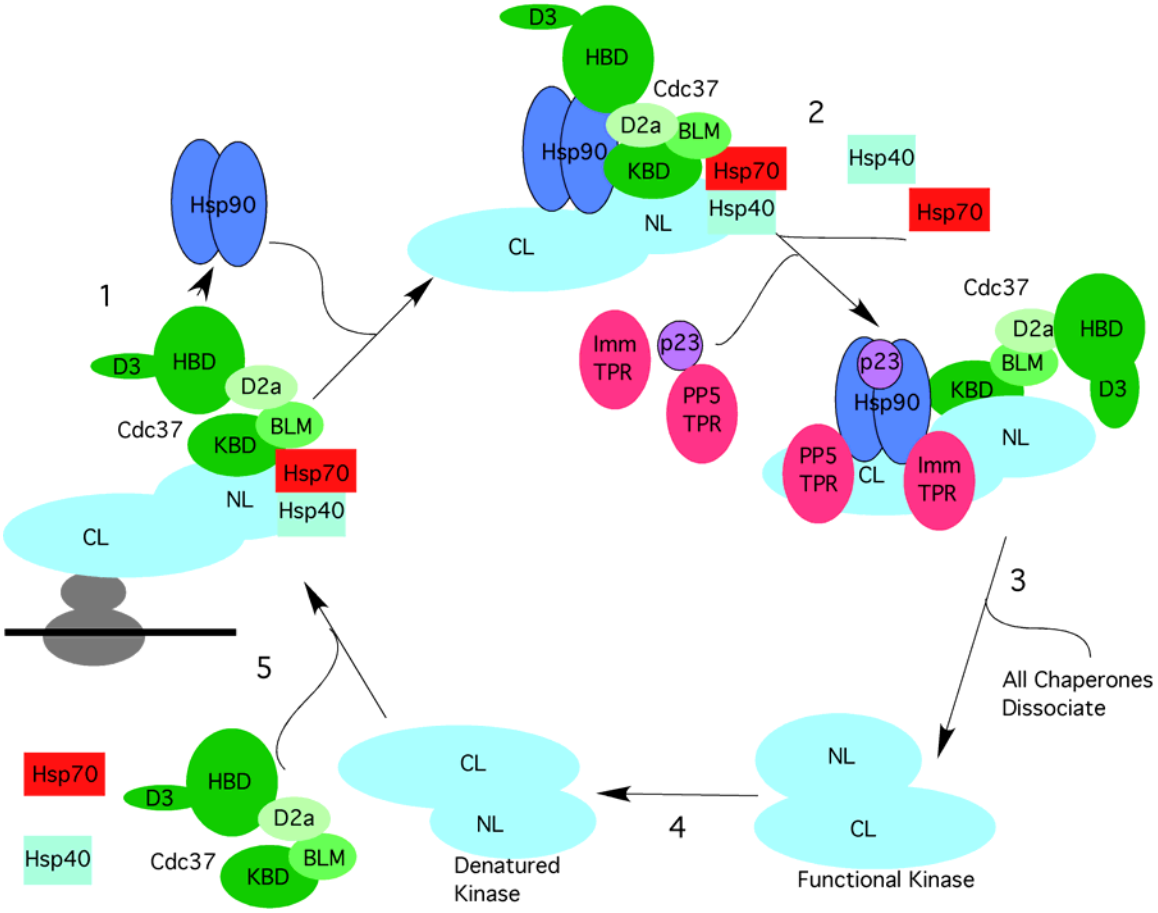


Fig. 27. **Cdc37 modified Hsp70/Hsp90 Chaperone Cycle. (1)** As the protein kinase emerges from the synthesizing ribosome, Cdc37 binds to the NL of the kinase with its KBD while at the same time Hsp40 and Hsp70 recognize and bind the nascent polypeptide. Hsp90 is recruited into this "early complex" also during this time through the HBD of Cdc37. **(2)** Once the supposed "intermediate complex" is assembled, Cdc37 induces Hsp70 and Hsp40 to dissociate from the complex via Cdc37's Bag-like motif (BLM). While possibly at the same time, Cdc37 causes Hsp90 to bind ATP and tightly bind the client kinase. This is followed by the association of the "late complex" co-chaperones. **(3)** Eventually, Hsp90 hydrolyzes the bound ATP to ADP and releases the functional kinase. **(4)** Possibly after sometime, the client kinase becomes unstable. (5) Cdc37 recognizes the unstable client kinase and binds it, in the process re-recruiting it back into the Hsp70/Hsp90 chaperone cycle.

referred to as D2a; and interestingly its deletion from constructs of Cdc37's Hsp90 binding domain (D2) causes the construct to bind Hsp90 stronger in the presence of GA and weaker in its absence (*110*). This observation suggests this motif in Cdc37 and Hop may play some role in modulating nucleotide-mediated conformational change, which in turn modulates client binding.

A speculative model for Cdc37's participation in Hsp90's chaperone cycle could be described accordingly (Fig. 27). Cdc37 recognizes and binds the NL of the nascent kinase polypeptide as it emerges from the ribosome. This possibly occurs along with Hsp40 and Hsp70 binding to the polypeptide. Hsp90 is recruited via the HBD of Cdc37, while Cdc37 also interacts with Hsp70 through its Bag-like motif. Cdc37 then stimulates Hsp70 to release the client kinase, while Cdc37's D2a domain activates the nucleotide mediated conformational switching of Hsp90 and subsequent client kinase binding by Cdc37's KBD. "Late complex" co-chaperones, such as p23 and PP5 then bind to the dimerized Hsp90 chaperone complex and help facilitate the folding of the client kinase.

Hsp90 Client Binding Mechanisms

The findings in this report indicate that Hsp90 interacts with kinases and possibly other clients at two different sites. These findings combined with the possibility that Hsp90 may have more than one client binding interface or client binding motif (CBM) suggests a relatively novel Hsp90 mechanism of action. The NAD and MD have both been shown to have some form of client binding activity indicating that each one possesses a binding interface. Besides having binding interfaces, these domains also contain most of the residues needed for Hsp90 ATPase activity. The NAD contains the

ATP binding pocket, and the MD possesses several critical residues needed for ATP hydrolysis [R380 and Q384 in yeast] (*54*). However, these catalytic residues, like the binding interfaces, are separated by the evolutionarily variable and extremely flexible LD. The organization of Hsp90's structure suggests that in order for Hsp90 to hydrolyze ATP when it is in the ATP-bound or "slow on/slow off" conformation, it must bring the NAD and MD together, which in turn would bring the binding interfaces together. The approximated binding interfaces could then synergistically bind the client in a number of ways (Fig. 28): (i) combining the binding interfaces to create one large interface; (ii) "sandwiching" one region of the client between the two binding interfaces; or (iii) each binding interface recognizing a different site on the client before the domains are brought together upon ATP binding. The latter mechanism is the most favored as it truly utilizes both client sites and suggests that Hsp90 folds extended and unstable clients by bringing together their different domains. The CD has also been shown to possess client binding activity but was not fully considered when developing this simple model due to the complexity that may entail.

Additionally, two possibilities exist for the approximated orientation of the NAD and MD (Fig. 29): (i) the two domains interact in an intra-molecular manner or within the same Hsp90 subunit; or (ii) the two domains interact in an intermolecular manner with the NAD of one Hsp90 subunit interacting with MD of the other (suggested by Harris and co-workers, unpublished). Again, the latter mechanism is favored because it takes full advantage of the need for Hsp90 to dimerize for ATP hydrolysis to occur. Each model must also take into account that both Hsp90 monomers must place their NAD dimerization motifs (NDM) next to each other upon ATP binding and p23 association.

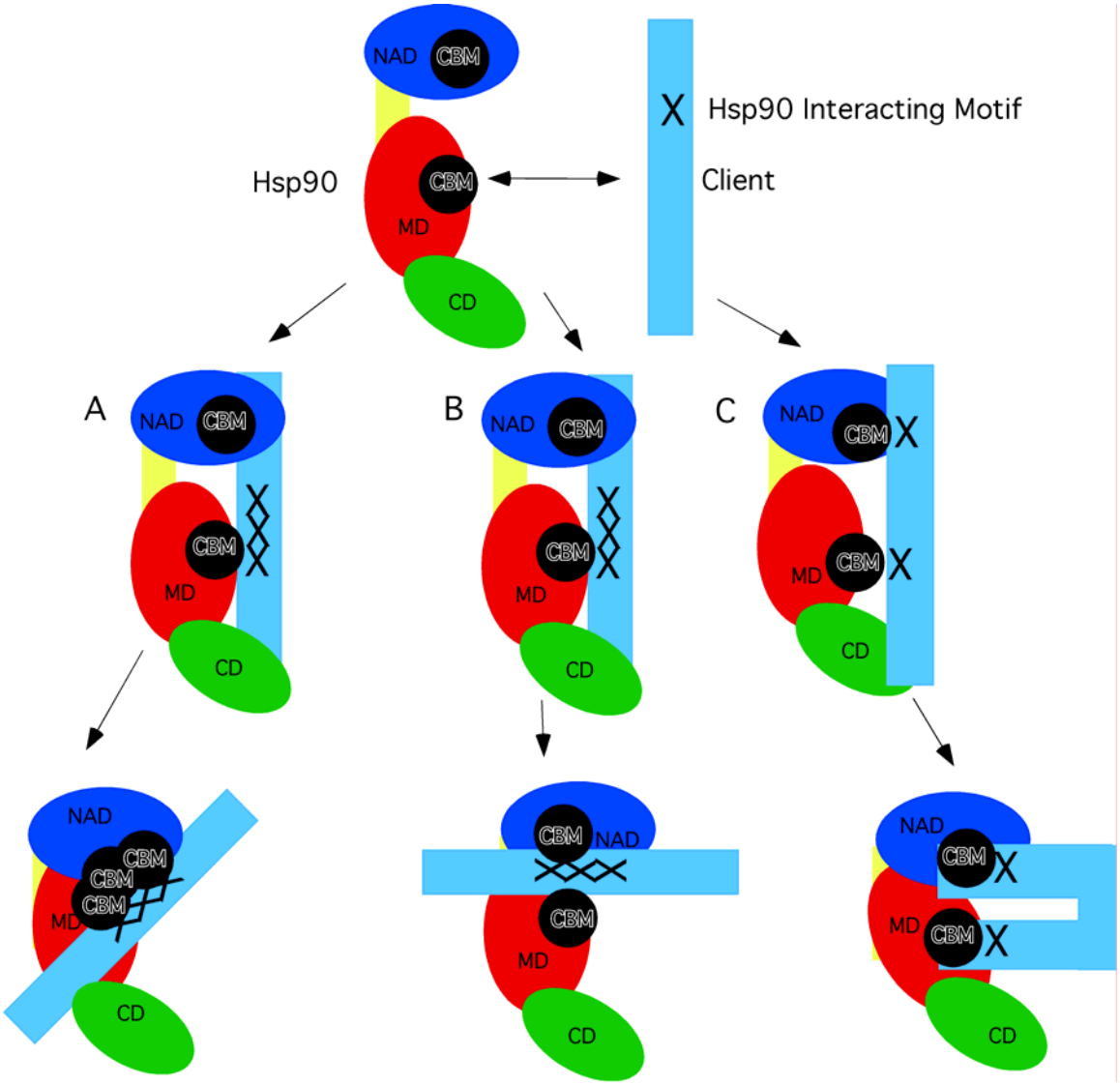


Fig. 28. **Models of Hsp90's dual client binding mechanism.** The black circles represent the client binding motifs (CBM) while all other Hsp90 domains are labeled and colored according to **Fig.2.** The light blue rectangle represents the client protein with each X indicating a possible Hsp90 binding site. **(A)** One or the other CBM weakly binds the client while in the open or ADP-bound conformation. Upon ATP-binding, the CBMs come together to form an enhanced CBM surface that strongly binds the client or provides a folding surface for clients to template off of. **(B)** Again, one or the other CBM weakly binds the client while in the open or ADP-bound conformation. Upon ATP binding, the CBMs come together to "sandwich" the client in a hydrophobic embrace. **(C)** Each CBM binds weakly to different binding sites on the client while in the ADP-bound conformation. Upon ATP binding, the CBMs come together bringing the client's binding sites along with them, in order to compress, collapse, and fold the client protein. Additional models that combine any of the three described models are also possible.

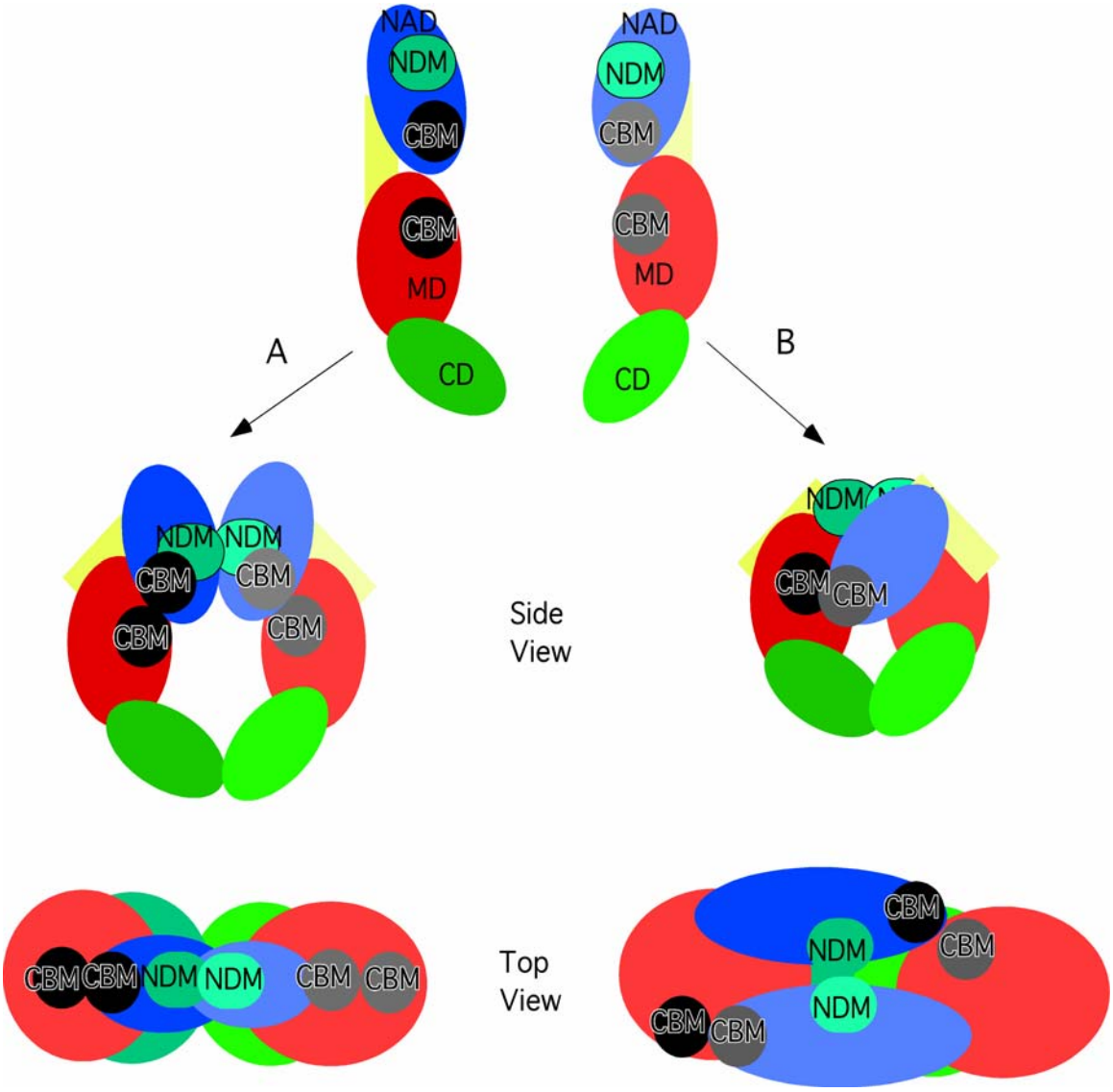


Fig. 29. **Model of Hsp90 dimerization.** The teal and cyan ovals represent the NAD dimerization motifs (NDM) while all other Hsp90 domains are labeled and colored according to **Fig.2.** and **Fig. 28.** In both models Hsp90 starts out in the ADP-bound incompletely dimerized conformation. Upon ATP-binding the NADs come together and the NDMs associate. While at the same time the NAD and MD come close, bringing together the CBMs. **(A)** The NADs and MDs and therefore the CBMs associate intra-molecularly. **(B)** One NAD associates with the other monomer's MD and vice versa, making the CBMs associate intermolecularly.

This overall mechanism would also require that the LD remain flexible, as the inability to do so would result in some form of inhibition of Hsp90's function. Coincidentally, all known post-translational modifications of Hsp90 have been mapped to the LD (*56*), long hinting that it is a major site of Hsp90 regulation.

Protein Kinase Evolution

The role of Hsp90 and Cdc37 in protein kinase folding appears to be the stabilization of structural motifs in protein kinases that have become exposed or unstable. These structural motifs are not defined by amino acid sequence and currently cannot be defined by any specific secondary or tertiary structure. This ambiguity, however difficult it makes the study of these interactions, suggests a vast amount versatility and functionality in the ability of Hsp90 and Cdc37 to chaperone their clients.

Alterations of flexible hinges or other functional motifs in kinases can induce or reduce chaperone association. As one would expect, point mutations that disrupt and destabilize the kinase structure, cause an increase in Hsp90 and Cdc37 association. Yet, the residues in these motifs do not necessarily define the area of Hsp90 or Cdc37 interaction, but instead may indicate the motif is required to stabilize another structural feature that is recognized by Hsp90 or Cdc37. Furthermore, the affinity of Hsp90 and Cdc37 for structurally unstable kinases may be by design, as some inherently unstable kinases only function with the aid of Hsp90 and Cdc37. This dependence on Hsp90 and Cdc37 is observed with the kinases v-Src and ErbB2 (*148, 171*), which rely on continuous chaperone association for their kinase activity and cellular stability. This continuous dependence on Hsp90 and Cdc37 has even developed into a form of

regulation for ErbB2 (*171*). Thus, given the assistance of Hsp90 and Cdc37, certain alterations that weaken the structural stability of kinases may be tolerated within the cell.

This assistance may have allowed protein kinases to evolve into the signaling proteins required for the viability of countless organisms. The crystal structure of the *Archaeoglobus fulgidus* kinase, Rio2 hints at this possibility (*202*). Evolutionarily conserved, a wide variety of homologs of Rio2 have been identified from Archaea to humans. Though its exact mode of action is not clear, Rio2 has been found to be required for rRNA processing in yeast (*203*). The structure of Rio2, described as atypical, is similar to most protein kinases except it does not have a truly complete CL and it has a winged helix domain sitting directly on top of the NL. The incomplete CL does not contain an APE box motif or structural motifs VIII-XI, but instead contains two alpha-helices following the DFG-like motif in subdomain VII. This suggests that Rio2 does not have a discernible substrate binding motif, activation loop, or most of the structural features found in the CL of typical kinases. What Rio2 does have is the minimum kinase structure shown by us to be required for Hsp90 association (*163*) (Fig. 30), this being the NL through subdomain VIB according to our findings. These features suggests that Hsp90 may have evolved to recognize the minimum structural features required for kinase activity since these subdomains most likely have always been present throughout kinase evolution. On the other hand, this might also suggest that kinases were allowed to evolve because their minimal structure was recognized by Hsp90.

The intimate interactions of the NL and the preceding winged helix domain of Rio2 indicates that Cdc37 would not be needed to provide stability while folding. Similar in function to the anti-parallel β-sheets of the NSD in MAPK/SAPKs, the

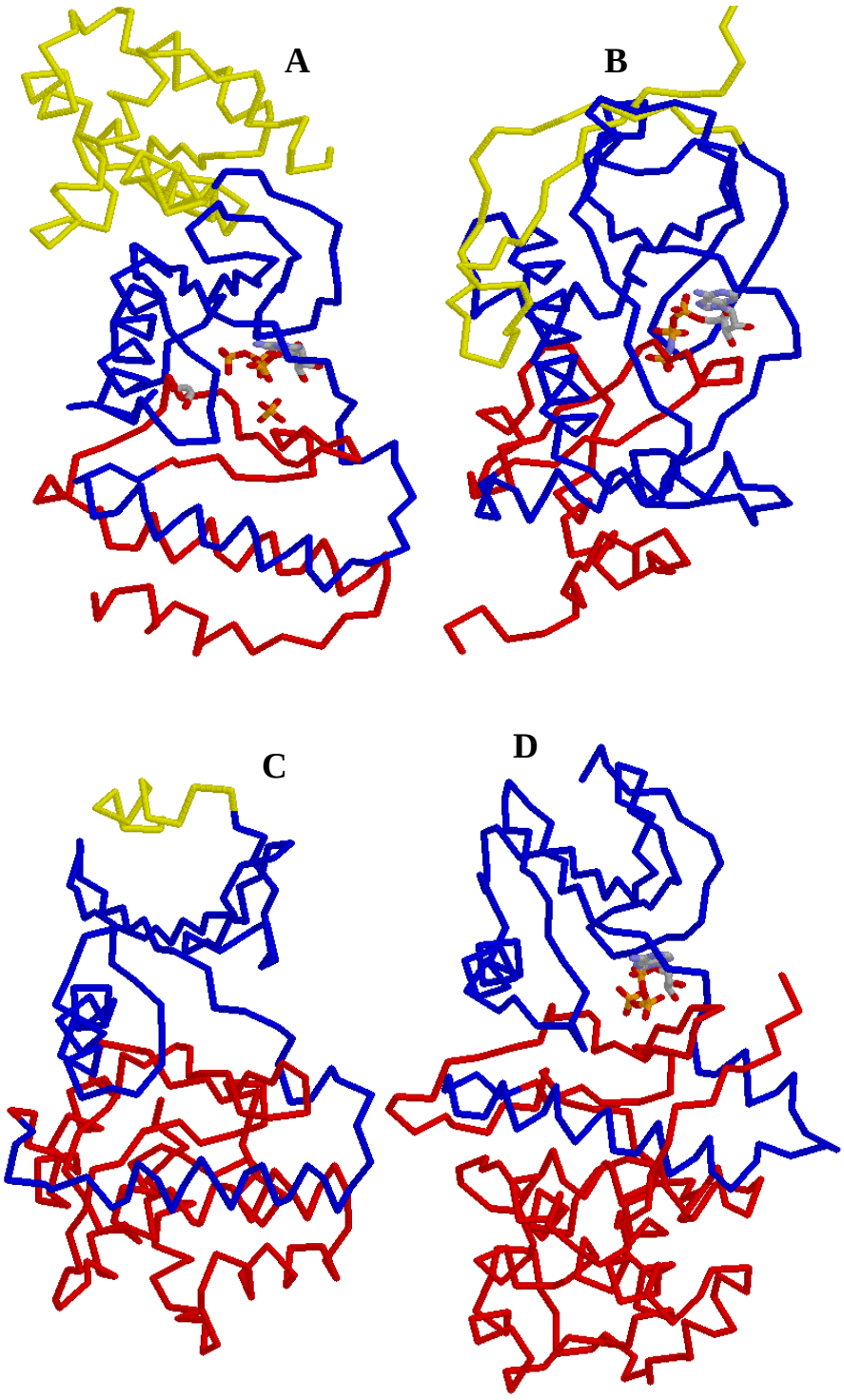


Fig. 30. **Strucural comparison of atypical and typical protein kinases.** Each kinase is shown with its ATP-binding cleft facing away from the viewer. N-terminal non-catalyic regions are shown in yellow. The defined Hsp90 and Cdc37 binding domain according to Prince and Matts (*163*) is shown in blue. The remaining CL structure is shown in red. **(A)** Rio2 (PDB: 1TQP) **(B)** ChaK (PDB: 1IA9) **(C)** APH(3')-IIIa (PDB: 1L8T) **(D)** Cdk2 (PDB: 1FIN)

winged helix domain instead places an α-helix directly on top of the five β-sheets of the NL to stabilize the structure, thus suggesting that the KBD of Cdc37 may instead utilize an alpha helix structure to stabilize the NL of kinases.

Additionally, although the structure of the NL of Rio2 is similar to typical kinases the truncated CL resembles the corresponding lobe of metabolic enzymes with the ATP-grasp fold. This is also found in another atypical kinase referred to as ChaK (*204*). This feature indicates, as suggested by Grishin, that protein kinases evolved from metabolic enzymes (*205*). This hypothesis is further strengthened by the crystal structure of APH(3')-IIIa; a kinase produced by pathogenic Gram-positive bacteria that confers broad antibiotic resistance by phosphorylating a variety of aminoglycosides (*206*). An activity most likely developed from the production of primary or secondary metabolites. The overall structure of APH(3')-IIIa is similar to Rio2 in that it has a preceding α-helix that layers on top of theβ -sheet structure of the NL. The rest of the NL structure resembles a typical protein kinase, while the CL similar to Rio2 and ChaK diverges after the capping of the αE-helix. Possibly reinforcing our findings, that this is the minimal structure needed to be recognized by Hsp90.

The evolution from single cell prokaryotes to multi-cellular eukaryotes in many instances may be dependent on groups of cells being able to communicate with each other. Protein kinase networks along with their receptors are in many ways designed for this communication. It therefore can be speculated that this ability to communicate may have started with APH(3')-IIIa-like proteins phosphorylating random metabolites which eventually evolved into protein kinases specifically adding phosphate groups to other proteins, in order to alter their function. It then could be further speculated, that the

ability of Hsp90 to maintain the function of these signaling proteins would indicate that Hsp90 and its homologues have played a major role in kinase evolution, and giving further support to the speculation of Lindquist and co-workers that Hsp90 is a vital vehicle for evolution at both the molecular and phenotypic level (*16, 19*).

REFERENCES

(1) Dinner, A. R., Sali, A., Smith, L. J., Dobson, C. M., and Karplus, M. (2000) Understanding protein folding via free-energy surfaces from theory and experiment. *Trends Biochem Sci 25*, 331-9.

(2) Dill, K. A., Bromberg, S., Yue, K., Fiebig, K. M., Yee, D. P., Thomas, P. D., and Chan, H. S. (1995) Principles of protein folding--a perspective from simple exact models. *Protein Sci 4*, 561-602.

(3) Anfinsen, C. B. (1973) Principles that govern the folding of protein chains. *Science 181*, 223-30.

(4) Zimmerman, S. B., and Trach, S. O. (1991) Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. *J Mol Biol 222*, 599-620.

(5) Jaenicke, R. (1987) Folding and association of proteins. *Prog Biophys Mol Biol 49*, 117-237.

(6) Buchner, J. (1996) Supervising the fold: functional principles of molecular chaperones. *Faseb J 10*, 10-9.

(7) Frydman, J., and Hartl, F. U. (1996) Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. *Science 272*, 1497-502.

(8) Ruddon, R. W., and Bedows, E. (1997) Assisted protein folding. *J Biol Chem 272*, 3125-8.

(9) Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004) Pathways of chaperone-mediated protein folding in the cytosol. *Nat Rev Mol Cell Biol 5*, 781-91.

(10) Frydman, J. (2001) Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu Rev Biochem 70*, 603-47.

(11) Hartl, F. U. (1996) Molecular chaperones in cellular protein folding. *Nature 381*, 571-9.

(12) Goetz, M. P., Toft, D. O., Ames, M. M., and Erlichman, C. (2003) The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann Oncol 14*, 1169-76.

(13) Lai, B. T., Chin, N. W., Stanek, A. E., Keh, W., and Lanks, K. W. (1984) Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. *Mol Cell Biol 4*, 2802-10.

(14) Wegele, H., Muller, L., and Buchner, J. (2004) Hsp70 and Hsp90--a relay team for protein folding. *Rev Physiol Biochem Pharmacol 151*, 1-44.

(15) Pratt, W. B., and Toft, D. O. (2003) Regulation of Signaling Protein Function and Trafficking by the hsp90/hsp70-Based Chaperone Machinery. *Experimental Biology and Medicine 228*, 111-133.

(16) Rutherford, S. L., and Lindquist, S. (1998) Hsp90 as a capacitor for morphological evolution. *Nature 396*, 336-42.

(17) Wagner, G. P., Chiu, C. H., and Hansen, T. F. (1999) Is Hsp90 a regulator of evolvability? *J Exp Zool 285*, 116-8.
(18) Yahara, I. (1999) The role of HSP90 in evolution. *Genes Cells 4*, 375-9.
(19) Queitsch, C., Sangster, T. A., and Lindquist, S. (2002) Hsp90 as a capacitor of phenotypic variation. *Nature 417*, 618-24.
(20) Blagosklonny, M. V. (2002) Hsp-90-associated oncoproteins: multiple targets of geldanamycin and its analogs. *Leukemia 16*, 455-62.
(21) Citri, A., Kochupurakkal, B. S., and Yarden, Y. (2004) The achilles heel of ErbB-2/HER2: regulation by the Hsp90 chaperone machine and potential for pharmacological intervention. *Cell Cycle 3*, 51-60.
(22) Neckers, L., and Ivy, S. P. (2003) Heat shock protein 90. *Curr Opin Oncol 15*, 419-24.
(23) Neckers, L. (2002) Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol Med 8*, S55-61.
(24) Kamal, A., Boehm, M. F., and Burrows, F. J. (2004) Therapeutic and diagnostic implications of Hsp90 activation. *Trends Mol Med 10*, 283-90.
(25) Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, F. J. (2003) A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature 425*, 407-10.
(26) Neckers, L., and Neckers, K. (2005) Heat-shock protein 90 inhibitors as novel cancer chemotherapeutics - an update. *Expert Opin Emerg Drugs 10*, 137-49.
(27) Goetz, M. P., Toft, D., Reid, J., Ames, M., Stensgard, B., Safgren, S., Adjei, A. A., Sloan, J., Atherton, P., Vasile, V., Salazaar, S., Adjei, A., Croghan, G., and Erlichman, C. (2005) Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. *J Clin Oncol 23*, 1078-87.
(28) Bardwell, J. C., and Craig, E. A. (1987) Eukaryotic Mr 83,000 heat shock protein has a homologue in Escherichia coli. *Proc Natl Acad Sci U S A 84*, 5177-81.
(29) Krone, P. H., Sass, J. B., and Lele, Z. (1997) Heat shock protein gene expression during embryonic development of the zebrafish. *Cell Mol Life Sci 53*, 122-9.
(30) Moore, S. K., Kozak, C., Robinson, E. A., Ullrich, S. J., and Appella, E. (1989) Murine 86- and 84-kDa heat shock proteins, cDNA sequences, chromosome assignments, and evolutionary origins. *J Biol Chem 264*, 5343-51.
(31) Sorger, P. K., and Pelham, H. R. (1987) The glucose-regulated protein grp94 is related to heat shock protein hsp90. *J Mol Biol 194*, 341-4.
(32) Felts, S. J., Owen, B. A., Nguyen, P., Trepel, J., Donner, D. B., and Toft, D. O. (2000) The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. *J Biol Chem 275*, 3305-12.
(33) Chen, C. F., Chen, Y., Dai, K., Chen, P. L., Riley, D. J., and Lee, W. H. (1996) A new member of the hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat shock. *Mol Cell Biol 16*, 4691-9.
(34) Radanyi, C., Renoir, J. M., Sabbah, M., and Baulieu, E. E. (1989) Chick heat-shock protein of Mr = 90,000, free or released from progesterone receptor, is in a dimeric form. *J Biol Chem 264*, 2568-73.

(35) Minami, Y., Kawasaki, H., Miyata, Y., Suzuki, K., and Yahara, I. (1991) Analysis of native forms and isoform compositions of the mouse 90-kDa heat shock protein, HSP90. *J Biol Chem 266*, 10099-103.
(36) Perdew, G. H., Hord, N., Hollenback, C. E., and Welsh, M. J. (1993) Localization and characterization of the 86- and 84-kDa heat shock proteins in Hepa 1c1c7 cells. *Exp Cell Res 209*, 350-6.
(37) Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell 89*, 239-50.
(38) Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell 90*, 65-75.
(39) Prodromou, C., Roe, S. M., Piper, P. W., and Pearl, L. H. (1997) A molecular clamp in the crystal structure of the N-terminal domain of the yeast Hsp90 chaperone. *Nat Struct Biol 4*, 477-82.
(40) Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem 42*, 260-6.
(41) Obermann, W. M., Sondermann, H., Russo, A. A., Pavletich, N. P., and Hartl, F. U. (1998) In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J Cell Biol 143*, 901-10.
(42) Jez, J. M., Chen, J. C., Rastelli, G., Stroud, R. M., and Santi, D. V. (2003) Crystal structure and molecular modeling of 17-DMAG in complex with human Hsp90. *Chem Biol 10*, 361-8.
(43) Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P. C., Nicolas, A., and Forterre, P. (1997) An atypical topoisomerase II from Archaea with implications for meiotic recombination. *Nature 386*, 414-7.
(44) Dutta, R., and Inouye, M. (2000) GHKL, an emergent ATPase/kinase superfamily. *Trends Biochem Sci 25*, 24-8.
(45) Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J. E., Roe, S. M., Piper, P. W., and Pearl, L. H. (2000) The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. *Embo J 19*, 4383-92.
(46) Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E. S., Litwack, G., and Toft, D. (1997) Nucleotides and two functional states of hsp90. *J Biol Chem 272*, 8007-12.
(47) Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J Biol Chem 274*, 17525-33.
(48) Hartson, S. D., Thulasiraman, V., Huang, W., Whitesell, L., and Matts, R. L. (1999) Molybdate inhibits hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. *Biochemistry 38*, 3837-49.
(49) Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *Embo J 17*, 4829-36.

(50) Scheibel, T., Neuhofen, S., Weikl, T., Mayr, C., Reinstein, J., Vogel, P. D., and Buchner, J. (1997) ATP-binding properties of human Hsp90. *J Biol Chem 272*, 18608-13.
(51) Weikl, T., Muschler, P., Richter, K., Veit, T., Reinstein, J., and Buchner, J. (2000) C-terminal regions of Hsp90 are important for trapping the nucleotide during the ATPase cycle. *J Mol Biol 303*, 583-92.
(52) Wegele, H., Muschler, P., Bunck, M., Reinstein, J., and Buchner, J. (2003) Dissection of the contribution of individual domains to the ATPase mechanism of Hsp90. *J Biol Chem 278*, 39303-10.
(53) Roe, S. M., Ali, M. M., Meyer, P., Vaughan, C. K., Panaretou, B., Piper, P. W., Prodromou, C., and Pearl, L. H. (2004) The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37). *Cell 116*, 87-98.
(54) Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S. M., Panaretou, B., Piper, P. W., and Pearl, L. H. (2003) Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions. *Mol Cell 11*, 647-58.
(55) Harris, S. F., Shiau, A. K., and Agard, D. A. (2004) The crystal structure of the carboxy-terminal dimerization domain of htpG, the Escherichia coli Hsp90, reveals a potential substrate binding site. *Structure (Camb) 12*, 1087-97.
(56) Dougherty, J. J., Rabideau, D. A., Iannotti, A. M., Sullivan, W. P., and Toft, D. O. (1987) Identification of the 90 kDa substrate of rat liver type II casein kinase with the heat shock protein which binds steroid receptors. *Biochim Biophys Acta 927*, 74-80.
(57) Lees-Miller, S. P., Chen, Y. R., and Anderson, C. W. (1990) Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. *Mol Cell Biol 10*, 6472-81.
(58) Meng, X., Devin, J., Sullivan, W. P., Toft, D., Baulieu, E. E., and Catelli, M. G. (1996) Mutational analysis of Hsp90 alpha dimerization and subcellular localization: dimer disruption does not impede "in vivo' interaction with estrogen receptor. *J Cell Sci 109 (Pt 7)*, 1677-87.
(59) Louvion, J. F., Warth, R., and Picard, D. (1996) Two eukaryote-specific regions of Hsp82 are dispensable for its viability and signal transduction functions in yeast. *Proc Natl Acad Sci U S A 93*, 13937-42.
(60) Meyer, P., Prodromou, C., Liao, C., Hu, B., Roe, S. M., Vaughan, C. K., Vlasic, I., Panaretou, B., Piper, P. W., and Pearl, L. H. (2004) Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery. *Embo J 23*, 1402-10.
(61) Odunuga, O. O., Longshaw, V. M., and Blatch, G. L. (2004) Hop: more than an Hsp70/Hsp90 adaptor protein. *Bioessays 26*, 1058-68.
(62) Hernandez, M. P., Sullivan, W. P., and Toft, D. O. (2002) The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. *J Biol Chem 277*, 38294-304.
(63) Johnson, B. D., Schumacher, R. J., Ross, E. D., and Toft, D. O. (1998) Hop modulates Hsp70/Hsp90 interactions in protein folding. *J Biol Chem 273*, 3679-86.

(64) Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell 101*, 199-210.

(65) Elhyany, S., Assa-Kunik, E., Tsory, S., Muller, T., Fedida, S., Segal, S., and Fishman, D. (2004) The integrity of cholesterol-enriched microdomains is essential for the constitutive high activity of protein kinase B in tumour cells. *Biochem Soc Trans 32*, 837-9.

(66) Marcu, M. G., Chadli, A., Bouhouche, I., Catelli, M., and Neckers, L. M. (2000) The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. *J Biol Chem 275*, 37181-6.

(67) Soti, C., Racz, A., and Csermely, P. (2002) A Nucleotide-dependent molecular switch controls ATP binding at the C-terminal domain of Hsp90. N-terminal nucleotide binding unmasks a C-terminal binding pocket. *J Biol Chem 277*, 7066-75.

(68) Yun, B. G., Huang, W., Leach, N., Hartson, S. D., and Matts, R. L. (2004) Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions. *Biochemistry 43*, 8217-29.

(69) Erbse, A., Mayer, M. P., and Bukau, B. (2004) Mechanism of substrate recognition by Hsp70 chaperones. *Biochem Soc Trans 32*, 617-21.

(70) Mayer, M. P., and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci 62*, 670-84.

(71) Thulasiraman, V., Yun, B. G., Uma, S., Gu, Y., Scroggins, B. T., and Matts, R. L. (2002) Differential inhibition of Hsc70 activities by two Hsc70-binding peptides. *Biochemistry 41*, 3742-53.

(72) Fan, C. Y., Lee, S., and Cyr, D. M. (2003) Mechanisms for regulation of Hsp70 function by Hsp40. *Cell Stress Chaperones 8*, 309-16.

(73) Shomura, Y., Dragovic, Z., Chang, H. C., Tzvetkov, N., Young, J. C., Brodsky, J. L., Guerriero, V., Hartl, F. U., and Bracher, A. (2005) Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange. *Mol Cell 17*, 367-79.

(74) Bimston, D., Song, J., Winchester, D., Takayama, S., Reed, J. C., and Morimoto, R. I. (1998) BAG-1, a negative regulator of Hsp70 chaperone activity, uncouples nucleotide hydrolysis from substrate release. *Embo J 17*, 6871-8.

(75) Sondermann, H., Scheufler, C., Schneider, C., Hohfeld, J., Hartl, F. U., and Moarefi, I. (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science 291*, 1553-7.

(76) Nollen, E. A., Kabakov, A. E., Brunsting, J. F., Kanon, B., Hohfeld, J., and Kampinga, H. H. (2001) Modulation of in vivo HSP70 chaperone activity by Hip and Bag-1. *J Biol Chem 276*, 4677-82.

(77) Smith, D. F., Stensgard, B. A., Welch, W. J., and Toft, D. O. (1992) Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. *J Biol Chem 267*, 1350-6.

(78) Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsu, K., Madden, B., McCormick, D. J., and Toft, D. O. (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol Cell Biol 13*, 869-76.
(79) Chen, S., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. *Mol Endocrinol 10*, 682-93.
(80) Gross, M., and Hessefort, S. (1996) Purification and characterization of a 66-kDa protein from rabbit reticulocyte lysate which promotes the recycling of hsp 70. *J Biol Chem 271*, 16833-41.
(81) Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. *Embo J 18*, 754-62.
(82) Johnson, J. L., Beito, T. G., Krco, C. J., and Toft, D. O. (1994) Characterization of a novel 23-kilodalton protein of unactive progesterone receptor complexes. *Mol Cell Biol 14*, 1956-63.
(83) Johnson, J. L., and Toft, D. O. (1994) A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. *J Biol Chem 269*, 24989-93.
(84) Weaver, A. J., Sullivan, W. P., Felts, S. J., Owen, B. A., and Toft, D. O. (2000) Crystal structure and activity of human p23, a heat shock protein 90 co-chaperone. *J Biol Chem 275*, 23045-52.
(85) Felts, S. J., and Toft, D. O. (2003) p23, a simple protein with complex activities. *Cell Stress Chaperones 8*, 108-13.
(86) Zhu, S., and Tytgat, J. (2004) Evolutionary epitopes of Hsp90 and p23: implications for their interaction. *Faseb J 18*, 940-7.
(87) Young, J. C., and Hartl, F. U. (2000) Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. *Embo J 19*, 5930-40.
(88) Pratt, W. B. (1998) The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc Soc Exp Biol Med 217*, 420-34.
(89) Pratt, W. B., and Toft, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev 18*, 306-60.
(90) Shao, J., Hartson, S. D., and Matts, R. L. (2002) Evidence that protein phosphatase 5 functions to negatively modulate the maturation of the Hsp90-dependent heme-regulated eIF2alpha kinase. *Biochemistry 41*, 6770-9.
(91) Nathan, D. F., Vos, M. H., and Lindquist, S. (1999) Identification of SSF1, CNS1, and HCH1 as multicopy suppressors of a Saccharomyces cerevisiae Hsp90 loss-of-function mutation. *Proc Natl Acad Sci U S A 96*, 1409-14.
(92) Panaretou, B., Siligardi, G., Meyer, P., Maloney, A., Sullivan, J. K., Singh, S., Millson, S. H., Clarke, P. A., Naaby-Hansen, S., Stein, R., Cramer, R., Mollapour, M., Workman, P., Piper, P. W., Pearl, L. H., and Prodromou, C. (2002) Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. *Mol Cell 10*, 1307-18.

(93) Lotz, G. P., Lin, H., Harst, A., and Obermann, W. M. (2003) Aha1 binds to the middle domain of Hsp90, contributes to client protein activation, and stimulates the ATPase activity of the molecular chaperone. *J Biol Chem 278*, 17228-35.
(94) Harst, A., Lin, H., and Obermann, W. M. (2005) Aha1 competes with Hop, p50 and p23 for binding to the molecular chaperone Hsp90 and contributes to kinase and hormone receptor activation. *Biochem J 387*, 789-96.
(95) Siligardi, G., Hu, B., Panaretou, B., Piper, P. W., Pearl, L. H., and Prodromou, C. (2004) Co-chaperone regulation of conformational switching in the Hsp90 ATPase cycle. *J Biol Chem 279*, 51989-98.
(96) MacLean, M., and Picard, D. (2003) Cdc37 goes beyond Hsp90 and kinases. *Cell Stress Chaperones 8*, 114-9.
(97) Pearl, L. H. (2005) Hsp90 and Cdc37 -- a chaperone cancer conspiracy. *Curr Opin Genet Dev 15*, 55-61.
(98) Reed, S. I. (1980) The selection of S. cerevisiae mutants defective in the start event of cell division. *Genetics 95*, 561-77.
(99) Brugge, J. S. (1986) Interaction of the Rous sarcoma virus protein pp60src with the cellular proteins pp50 and pp90. *Curr Top Microbiol Immunol 123*, 1-22.
(100) Gerber, M. R., Farrell, A., Deshaies, R. J., Herskowitz, I., and Morgan, D. O. (1995) Cdc37 is required for association of the protein kinase Cdc28 with G1 and mitotic cyclins. *Proc Natl Acad Sci U S A 92*, 4651-5.
(101) Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., and Ahringer, J. (2003) Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature 421*, 231-7.
(102) Cutforth, T., and Rubin, G. M. (1994) Mutations in Hsp83 and cdc37 impair signaling by the sevenless receptor tyrosine kinase in Drosophila. *Cell 77*, 1027-36.
(103) Schwarze, S. R., Fu, V. X., and Jarrard, D. F. (2003) Cdc37 enhances proliferation and is necessary for normal human prostate epithelial cell survival. *Cancer Res 63*, 4614-9.
(104) Stepanova, L., Yang, G., DeMayo, F., Wheeler, T. M., Finegold, M., Thompson, T. C., and Harper, J. W. (2000) Induction of human Cdc37 in prostate cancer correlates with the ability of targeted Cdc37 expression to promote prostatic hyperplasia. *Oncogene 19*, 2186-93.
(105) Stepanova, L., Finegold, M., DeMayo, F., Schmidt, E. V., and Harper, J. W. (2000) The oncoprotein kinase chaperone CDC37 functions as an oncogene in mice and collaborates with both c-myc and cyclin D1 in transformation of multiple tissues. *Mol Cell Biol 20*, 4462-73.
(106) Shao, J., Grammatikakis, N., Scroggins, B. T., Uma, S., Huang, W., Chen, J. J., Hartson, S. D., and Matts, R. L. (2001) Hsp90 regulates p50(cdc37) function during the biogenesis of the activeconformation of the heme-regulated eIF2 alpha kinase. *J Biol Chem 276*, 206-14.
(107) Hartson, S. D., Irwin, A. D., Shao, J., Scroggins, B. T., Volk, L., Huang, W., and Matts, R. L. (2000) p50(cdc37) is a nonexclusive Hsp90 cohort which participates intimately in Hsp90-mediated folding of immature kinase molecules. *Biochemistry 39*, 7631-44.

(108) Scroggins, B. T., Prince, T., Shao, J., Uma, S., Huang, W., Guo, Y., Yun, B. G., Hedman, K., Matts, R. L., and Hartson, S. D. (2003) High affinity binding of Hsp90 is triggered by multiple discrete segments of its kinase clients. *Biochemistry 42*, 12550-61.
(109) Siligardi, G., Panaretou, B., Meyer, P., Singh, S., Woolfson, D. N., Piper, P. W., Pearl, L. H., and Prodromou, C. (2002) Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. *J Biol Chem 277*, 20151-9.
(110) Shao, J., Irwin, A., Hartson, S. D., and Matts, R. L. (2003) Functional dissection of cdc37: characterization of domain structure and amino acid residues critical for protein kinase binding. *Biochemistry 42*, 12577-88.
(111) Grammatikakis, N., Lin, J. H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. *Mol Cell Biol 19*, 1661-72.
(112) Rao, J., Lee, P., Benzeno, S., Cardozo, C., Albertus, J., Robins, D. M., and Caplan, A. J. (2001) Functional interaction of human Cdc37 with the androgen receptor but not with the glucocorticoid receptor. *J Biol Chem 276*, 5814-20.
(113) Bandhakavi, S., McCann, R. O., Hanna, D. E., and Glover, C. V. (2003) Genetic interactions among ZDS1,2, CDC37, and protein kinase CK2 in Saccharomyces cerevisiae. *FEBS Lett 554*, 295-300.
(114) Glover, C. V., 3rd. (1998) On the physiological role of casein kinase II in Saccharomyces cerevisiae. *Prog Nucleic Acid Res Mol Biol 59*, 95-133.
(115) Scholz, G. M., Cartledge, K., and Hall, N. E. (2001) Identification and characterization of Harc, a novel Hsp90-associating relative of Cdc37. *J Biol Chem 276*, 30971-9.
(116) Wang, X., Grammatikakis, N., and Hu, J. (2002) Role of p50/CDC37 in hepadnavirus assembly and replication. *J Biol Chem 277*, 24361-7.
(117) Smith, D. F. (1993) Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes. *Mol Endocrinol 7*, 1418-29.
(118) Yorgin, P. D., Hartson, S. D., Fellah, A. M., Scroggins, B. T., Huang, W., Katsanis, E., Couchman, J. M., Matts, R. L., and Whitesell, L. (2000) Effects of geldanamycin, a heat-shock protein 90-binding agent, on T cell function and T cell nonreceptor protein tyrosine kinases. *J Immunol 164*, 2915-23.
(119) Benton, P. A., Barrett, D. J., Matts, R. L., and Lloyd, R. E. (1996) The outcome of poliovirus infections in K562 cells is cytolytic rather than persistent after hemin-induced differentiation. *J Virol 70*, 5525-32.
(120) Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science 298*, 1912-34.
(121) Cohen, P. (2002) Protein kinases--the major drug targets of the twenty-first century? *Nat Rev Drug Discov 1*, 309-15.
(122) Fischer, P. M. (2004) The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases. *Curr Med Chem 11*, 1563-83.
(123) Hanks, S. K., and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *Faseb J 9*, 576-96.

(124) Hartson, S. D., Barrett, D. J., Burn, P., and Matts, R. L. (1996) Hsp90-mediated folding of the lymphoid cell kinase p56lck. *Biochemistry 35*, 13451-9.
(125) Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes Dev 10*, 1491-502.
(126) Mort-Bontemps-Soret, M., Facca, C., and Faye, G. (2002) Physical interaction of Cdc28 with Cdc37 in Saccharomyces cerevisiae. *Mol Genet Genomics 267*, 447-58.
(127) Palacios, E. H., and Weiss, A. (2004) Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene 23*, 7990-8000.
(128) Obaya, A. J., and Sedivy, J. M. (2002) Regulation of cyclin-Cdk activity in mammalian cells. *Cell Mol Life Sci 59*, 126-42.
(129) De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S. H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature 363*, 595-602.
(130) Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell 103*, 239-52.
(131) Xie, X., Gu, Y., Fox, T., Coll, J. T., Fleming, M. A., Markland, W., Caron, P. R., Wilson, K. P., and Su, M. S. (1998) Crystal structure of JNK3: a kinase implicated in neuronal apoptosis. *Structure 6*, 983-91.
(132) Sato, S., Fujita, N., and Tsuruo, T. (2000) Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A 97*, 10832-7.
(133) Fujita, N., Sato, S., Ishida, A., and Tsuruo, T. (2002) Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. *J Biol Chem 277*, 10346-53.
(134) Picard, D. (2002) Heat-shock protein 90, a chaperone for folding and regulation. *Cell Mol Life Sci 59*, 1640-8.
(135) Richter, K., and Buchner, J. (2001) Hsp90: chaperoning signal transduction. *J Cell Physiol 188*, 281-90.
(136) Caamano, C. A., Morano, M. I., Dalman, F. C., Pratt, W. B., and Akil, H. (1998) A conserved proline in the hsp90 binding region of the glucocorticoid receptor is required for hsp90 heterocomplex stabilization and receptor signaling. *J Biol Chem 273*, 20473-80.
(137) Giannoukos, G., Silverstein, A. M., Pratt, W. B., and Simons, S. S., Jr. (1999) The seven amino acids (547-553) of rat glucocorticoid receptor required for steroid and hsp90 binding contain a functionally independent LXXLL motif that is critical for steroid binding. *J Biol Chem 274*, 36527-36.
(138) Kaul, S., Murphy, P. J., Chen, J., Brown, L., Pratt, W. B., and Simons, S. S., Jr. (2002) Mutations at positions 547-553 of rat glucocorticoid receptors reveal that hsp90 binding requires the presence, but not defined composition, of a seven-amino acid sequence at the amino terminus of the ligand binding domain. *J Biol Chem 277*, 36223-32.
(139) Xu, M., Dittmar, K. D., Giannoukos, G., Pratt, W. B., and Simons, S. S., Jr. (1998) Binding of hsp90 to the glucocorticoid receptor requires a specific 7-amino acid sequence at the amino terminus of the hormone-binding domain. *J Biol Chem 273*, 13918-24.

(140) Couette, B., Jalaguier, S., Hellal-Levy, C., Lupo, B., Fagart, J., Auzou, G., and Rafestin-Oblin, M. E. (1998) Folding requirements of the ligand-binding domain of the human mineralocorticoid receptor. *Mol Endocrinol 12*, 855-63.
(141) Zhao, Q., Boschelli, F., Caplan, A. J., and Arndt, K. T. (2004) Identification of a conserved sequence motif that promotes Cdc37 and cyclin D1 binding to Cdk4. *J Biol Chem 279*, 12560-4.
(142) Scheibel, T., and Buchner, J. (1998) The Hsp90 complex--a super-chaperone machine as a novel drug target. *Biochem Pharmacol 56*, 675-82.
(143) Dalman, F. C., Scherrer, L. C., Taylor, L. P., Akil, H., and Pratt, W. B. (1991) Localization of the 90-kDa heat shock protein-binding site within the hormone-binding domain of the glucocorticoid receptor by peptide competition. *J Biol Chem 266*, 3482-90.
(144) Kimura, Y., Rutherford, S. L., Miyata, Y., Yahara, I., Freeman, B. C., Yue, L., Morimoto, R. I., and Lindquist, S. (1997) Cdc37 is a molecular chaperone with specific functions in signal transduction. *Genes Dev 11*, 1775-85.
(145) Scholz, G., Hartson, S. D., Cartledge, K., Hall, N., Shao, J., Dunn, A. R., and Matts, R. L. (2000) p50(Cdc37) can buffer the temperature-sensitive properties of a mutant of Hck. *Mol Cell Biol 20*, 6984-95.
(146) Scholz, G. M., Hartson, S. D., Cartledge, K., Volk, L., Matts, R. L., and Dunn, A. R. (2001) The molecular chaperone Hsp90 is required for signal transduction by wild-type Hck and maintenance of its constitutively active counterpart. *Cell Growth Differ 12*, 409-17.
(147) Shao, J., Prince, T., Hartson, S. D., and Matts, R. L. (2003) Phosphorylation of serine 13 is required for the proper function of the Hsp90 co-chaperone, Cdc37. *J Biol Chem 278*, 38117-20.
(148) Mimnaugh, E. G., Worland, P. J., Whitesell, L., and Neckers, L. M. (1995) Possible role for serine/threonine phosphorylation in the regulation of the heteroprotein complex between the hsp90 stress protein and the pp60v-src tyrosine kinase. *J Biol Chem 270*, 28654-9.
(149) Marcu, M. G., Schulte, T. W., and Neckers, L. (2000) Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J Natl Cancer Inst 92*, 242-8.
(150) Johnson, J. L., and Toft, D. O. (1995) Binding of p23 and hsp90 during assembly with the progesterone receptor. *Mol Endocrinol 9*, 670-8.
(151) Thulasiraman, V., and Matts, R. L. (1996) Effect of geldanamycin on the kinetics of chaperone-mediated renaturation of firefly luciferase in rabbit reticulocyte lysate. *Biochemistry 35*, 13443-50.
(152) Krieg, P. A., and Melton, D. A. (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res 12*, 7057-70.
(153) Hartson, S. D., and Matts, R. L. (1994) Association of Hsp90 with cellular Src-family kinases in a cell-free system correlates with altered kinase structure and function. *Biochemistry 33*, 8912-20.
(154) Bamberger, C. M., Wald, M., Bamberger, A. M., and Schulte, H. M. (1997) Inhibition of mineralocorticoid and glucocorticoid receptor function by the heat shock protein 90-binding agent geldanamycin. *Mol Cell Endocrinol 131*, 233-40.

(155) Chen, G., Cao, P., and Goeddel, D. V. (2002) TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. *Mol Cell 9*, 401-10.
(156) Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M., and Pratt, W. B. (1998) p50(cdc37) binds directly to the catalytic domain of Raf as well as to a site on hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. *J Biol Chem 273*, 20090-5.
(157) Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P., and Rosen, N. (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem 277*, 39858-66.
(158) Xu, W., Harrison, S. C., and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src. *Nature 385*, 595-602.
(159) Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell 3*, 629-38.
(160) Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999) Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. *Mol Cell 3*, 639-48.
(161) LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J., and Miller, W. T. (1998) Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue. *J Biol Chem 273*, 32129-34.
(162) Williams, J. C., Wierenga, R. K., and Saraste, M. (1998) Insights into Src kinase functions: structural comparisons. *Trends Biochem Sci 23*, 179-84.
(163) Prince, T., and Matts, R. L. (2004) Definition of protein kinase sequence motifs that trigger high affinity binding of Hsp90 and Cdc37. *J Biol Chem 279*, 39975-81.
(164) Fisher, R. P., and Morgan, D. O. (1994) A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell 78*, 713-24.
(165) Aligue, R., Akhavan-Niak, H., and Russell, P. (1994) A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *Embo J 13*, 6099-106.
(166) McGowan, C. H., and Russell, P. (1993) Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *Embo J 12*, 75-85.
(167) Parker, L. L., Atherton-Fessler, S., and Piwnica-Worms, H. (1992) p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. *Proc Natl Acad Sci U S A 89*, 2917-21.
(168) Yun, B. G., and Matts, R. L. (2005) Differential effects of Hsp90 inhibition on protein kinases regulating signal transduction pathways required for myoblast differentiation. *Exp Cell Res 307*, 212-23.
(169) Farrell, A., and Morgan, D. O. (2000) Cdc37 promotes the stability of protein kinases Cdc28 and Cak1. *Mol Cell Biol 20*, 749-54.
(170) Prince, T., Shao, J., Matts, R. L., and Hartson, S. D. (2005) Evidence for chaperone heterocomplexes containing both Hsp90 and VCP. *Biochem Biophys Res Commun 331*, 1331-7.
(171) Xu, W., Mimnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by

its kinase domain and is mediated by the chaperone protein Hsp90. *J Biol Chem 276*, 3702-8.
(172) Schulte, T. W., An, W. G., and Neckers, L. M. (1997) Geldanamycin-induced destabilization of Raf-1 involves the proteasome. *Biochem Biophys Res Commun 239*, 655-9.
(173) Bijlmakers, M. J., and Marsh, M. (2000) Hsp90 is essential for the synthesis and subsequent membrane association, but not the maintenance, of the Src-kinase p56(lck). *Mol Biol Cell 11*, 1585-95.
(174) Morgan, D. O. (1995) Principles of CDK regulation. *Nature 374*, 131-4.
(175) Wan, P. T., Garnett, M. J., Roe, S. M., Lee, S., Niculescu-Duvaz, D., Good, V. M., Jones, C. M., Marshall, C. J., Springer, C. J., Barford, D., and Marais, R. (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell 116*, 855-67.
(176) Xu, W., Yuan, X., Xiang, Z., Mimnaugh, E., Marcu, M., and Neckers, L. (2005) Surface charge and hydrophobicity determine ErbB2 binding to the Hsp90 chaperone complex. *Nat Struct Mol Biol 12*, 120-6.
(177) Yun, B. G., and Matts, R. L. (2005) Hsp90 functions to balance the phosphorylation state of Akt during C2C12 myoblast differentiation. *Cell Signal*.
(178) Seger, R., and Krebs, E. G. (1995) The MAPK signaling cascade. *Faseb J 9*, 726-35.
(179) Schulte, T. W., Blagosklonny, M. V., Romanova, L., Mushinski, J. F., Monia, B. P., Johnston, J. F., Nguyen, P., Trepel, J., and Neckers, L. M. (1996) Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf-1-MEK-mitogen-activated protein kinase signalling pathway. *Mol Cell Biol 16*, 5839-45.
(180) Tatebe, H., and Shiozaki, K. (2003) Identification of Cdc37 as a novel regulator of the stress-responsive mitogen-activated protein kinase. *Mol Cell Biol 23*, 5132-42.
(181) Zhang, H., Wu, W., Du, Y., Santos, S. J., Conrad, S. E., Watson, J. T., Grammatikakis, N., and Gallo, K. A. (2004) Hsp90/p50cdc37 is required for mixed-lineage kinase (MLK) 3 signaling. *J Biol Chem 279*, 19457-63.
(182) Miyata, Y., Ikawa, Y., Shibuya, M., and Nishida, E. (2001) Specific association of a set of molecular chaperones including HSP90 and Cdc37 with MOK, a member of the mitogen-activated protein kinase superfamily. *J Biol Chem 276*, 21841-8.
(183) Wilson, K. P., Fitzgibbon, M. J., Caron, P. R., Griffith, J. P., Chen, W., McCaffrey, P. G., Chambers, S. P., and Su, M. S. (1996) Crystal structure of p38 mitogen-activated protein kinase. *J Biol Chem 271*, 27696-700.
(184) Zhang, J., Zhang, F., Ebert, D., Cobb, M. H., and Goldsmith, E. J. (1995) Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. *Structure 3*, 299-307.
(185) Cobb, M. H., and Goldsmith, E. J. (1995) How MAP kinases are regulated. *J Biol Chem 270*, 14843-6.
(186) Tanoue, T., and Nishida, E. (2002) Docking interactions in the mitogen-activated protein kinase cascades. *Pharmacol Ther 93*, 193-202.

(187) Eblen, S. T., Catling, A. D., Assanah, M. C., and Weber, M. J. (2001) Biochemical and biological functions of the N-terminal, noncatalytic domain of extracellular signal-regulated kinase 2. *Mol Cell Biol 21*, 249-59.
(188) Mooney, L. M., and Whitmarsh, A. J. (2004) Docking interactions in the c-Jun N-terminal kinase pathway. *J Biol Chem 279*, 11843-52.
(189) Biondi, R. M., and Nebreda, A. R. (2003) Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem J 372*, 1-13.
(190) Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B., and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo J 15*, 2760-70.
(191) Sreedhar, A. S., Soti, C., and Csermely, P. (2004) Inhibition of Hsp90: a new strategy for inhibiting protein kinases. *Biochim Biophys Acta 1697*, 233-42.
(192) Zhang, H., and Burrows, F. (2004) Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med 82*, 488-99.
(193) Vanden Berghe, T., Kalai, M., van Loo, G., Declercq, W., and Vandenabeele, P. (2003) Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. *J Biol Chem 278*, 5622-9.
(194) Vasilevskaya, I. A., Rakitina, T. V., and O'Dwyer, P. J. (2004) Quantitative effects on c-Jun N-terminal protein kinase signaling determine synergistic interaction of cisplatin and 17-allylamino-17-demethoxygeldanamycin in colon cancer cell lines. *Mol Pharmacol 65*, 235-43.
(195) Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol 9*, 940-4.
(196) Yamaguchi, H., and Hendrickson, W. A. (1996) Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. *Nature 384*, 484-9.
(197) Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature 376*, 313-20.
(198) Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A., and Barford, D. (2002) Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Mol Cell 9*, 1227-40.
(199) Morris, M. C., Gondeau, C., Tainer, J. A., and Divita, G. (2002) Kinetic mechanism of activation of the Cdk2/cyclin A complex. Key role of the C-lobe of the Cdk. *J Biol Chem 277*, 23847-53.
(200) Giannini, A., and Bijlmakers, M. J. (2004) Regulation of the Src family kinase Lck by Hsp90 and ubiquitination. *Mol Cell Biol 24*, 5667-76.
(201) Romero, P., Obradovic, Z., and Dunker, A. K. (2004) Natively disordered proteins: functions and predictions. *Appl Bioinformatics 3*, 105-13.
(202) LaRonde-LeBlanc, N., and Wlodawer, A. (2004) Crystal structure of A. fulgidus Rio2 defines a new family of serine protein kinases. *Structure (Camb) 12*, 1585-94.
(203) Geerlings, T. H., Faber, A. W., Bister, M. D., Vos, J. C., and Raue, H. A. (2003) Rio2p, an evolutionarily conserved, low abundant protein kinase essential for processing of 20 S Pre-rRNA in Saccharomyces cerevisiae. *J Biol Chem 278*, 22537-45.

(204) Yamaguchi, H., Matsushita, M., Nairn, A. C., and Kuriyan, J. (2001) Crystal structure of the atypical protein kinase domain of a TRP channel with phosphotransferase activity. *Mol Cell 7*, 1047-57.
(205) Grishin, N. V. (1999) Phosphatidylinositol phosphate kinase: a link between protein kinase and glutathione synthase folds. *J Mol Biol 291*, 239-47.
(206) Fong, D. H., and Berghuis, A. M. (2002) Substrate promiscuity of an aminoglycoside antibiotic resistance enzyme via target mimicry. *Embo J 21*, 2323-31.
(207) All molecular images were made using Rasmol ucb 2.6.

VITA

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Thesis: DEFINED INTERACTIONS OF PROTEIN KINASES WITH HSP90 AND CDC37

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Scope and Method of Study: Protein kinases function to transduce the countless number of signals that allow a cell to grow and adapt to its environment. A significant number of protein kinases require assistance in being folded and converted into functional signal transducers from nascent polypeptides or in maintaining their functional conformation once folded. This assistance is provided by the molecular chaperone Hsp90 and its cohort Cdc37 which together function to fold protein kinases through a series of direct interactions: a dynamic process which remains poorly understood. Deletion and site specific mutagenesis was used to generate newly synthesized epitope tagged protein kinase constructs in a reticulocyte lysate coupled transcription and translation system. This was followed by immunoabsorption of the constructs and subsequent analysis of the co-absorbing endogenous Hsp90 and Cdc37.

Findings and Conclusions: In this work the interactions of three unique protein kinases with Hsp90 and Cdc37 were studied. Lck, a Src-family tyrosine kinase, which is widely known to interact with Hsp90 and Cdc37. Cdk2, a cyclin-dependent protein kinase, which has been found to genetically, although not physically, interact with Hsp90 and Cdc37. And JNK1α1, a stress activated protein kinase, which has been shown not to interact with either Hsp90 or Cdc37. The results of this work indicate that: (i) Hsp90 interacts with structural motifs found in both the N-terminal and C-terminal lobes protein kinases; (ii) Cdc37 interacts exclusively with the N-terminal lobe of protein kinases; (iii) depending on the kinase the presence of different structural motifs within the N-terminal lobe are required for Cdc37 interaction; and (iv) the minimum protein kinase structure required for high affinity salt stable Hsp90 and Cdc37 interaction is the N-terminal lobe through the stabilized αE-helix in the C-terminal lobe. In turn, these findings provide insight into how Hsp90 and Cdc37 recognize, bind and fold client protein kinases. In addition, these findings provide clues to the evolutionary relationship between the Hsp90 chaperone machine and protein kinases.

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