The yeast Cdc37 molecular chaperone: its association with Cak1 and its susceptibility to loss with oxidant and molybdate treatment

Thesis submitted by

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July 2014

Abstract

In eukaryotic cells the maturation and stabilization of newly-synthesised protein kinase molecules frequently requires the action of Cdc37, a molecular chaperone that will in turn often target these maturing protein kinase molecules onto another chaperone, Hsp90. Cdc37-nascent kinase associations are generally extremely transient in the environment of the living cell. Therefore they are seldom detectable by the yeast two hybrid system. This study investigated a Cdc37-kinase interaction that was detectable by two hybrid screening, the interaction of Cdc37 with the most atypical protein kinase of yeast, cyclin-dependent kinase (Cdk)-activating kinase (Cak1). It was shown that Cdc37 and Cak1 form a complex that is unaffected by Cdc37 phosphorylation status and sufficiently stable as to be isolatable by gel filtration.

Formation of this Cdc37/Cak1 complex was associated with a change to the phosphorylation status of Cak1. Mass spectrometry identified two new sites of Cak1 phosphorylation, Thr27, and Ser172. Yeast strains were constructed in which the sole, essential Cak1 has either a nonphosphorylatable or a phosphomimetic amino acid at these sites. These were then analysed for altered cell cycle progression and stress-sensitivity. Molybdate treatment of yeast was shown to cause the loss of Cdc37 and the fragmentation of Hsp90 at a discrete site.

Overall, the work in this thesis indicates that Cdc37 and Cak1 form a stable complex. Structural analysis of this complex may provide unique insight into how Cdc37 interacts with the N-terminal domain of protein kinases, also the unique structure of fungal Cak1 – a kinase which differs markedly from the Cak1 of human cells and is therefore potentially a promising antifungal drug target. The thesis also provides the first evidence for: (i) yeast Cak1 being regulated by phosphorylation; (ii) Cdc37 being both a determinant of and target

for oxidative stress resistance in yeast; and (iii) the molybdate anion inducing *in vivo* loss of Cdc37 and fragmentation of Hsp90.

Publications arising from this study: Millson SH, van Oosten-Hawle P, Alkuriji MA, Truman A, Siderius M, Piper PW. (2014) Cdc37 engages in stable, S14A mutation-reinforced association with the most atypical member of the yeast kinome, Cdk-activating kinase (Cak1). *Cell Stress Chaperones (in press).* PMID: 24452458

Acknowledgments

Firstly, my fulfilment would be possible without the support and help of Allah who bring the success to my study and my life.

I am indebted to some people who, in their presence, enabled me to undertake my PhD study successfully.

I would like to show my deep gratitude to my supervisors Professor Peter Piper and Dr Stefan Millson who provided me with the inspiration, encouragement and friendship throughout my study.

I would like also to thank my wife, my son, brothers and sisters, and my friends for their concerns and prayers.

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Abbreviations

5-FOA	5-fluoroorotic acid
17-AAG	17-allylamino, 17-demethoxygeldanamycin
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometres
aa	Amino acids
AD	Activation domain
ADP	Adenosine diphosphate
AMP-PNP	5'-adenylamido-diphosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BD	Binding domain
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
C. elegans	*Caenorhabditis elegans*
CK2	Casein kinase II
cm	centimetre
CTA domain	Carboxyl-terminal transcriptional activation domain
C-terminal	Carboxyl-terminal
dATP	Deoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dH_2O	Deionised water
D. melanogaster	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
dNTPs	Equimolar mixture of dATP, dTTP, dCTP, dGTP
DTT	Dithiothreiol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g	gram
GA	Geldanamycin
GC	General cell
GFP	Green fluorescent protein
GHKL	Gyrase, Hsp90, Histidine Kinase, MutL
h	Hour(s)
HF	High fidelity
HRP	Horseradish peroxidase
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
IMAC	Immobilised metal ion affinity chromatography
kb	Kilobase

kDa	Kilodalton
l	Litre
LB	Lysogeny broth
LiAc	Lithium acetate
M	Molar
mA	Milliamps
min	Minute
ml	Mililitre
mm	Milimetre
M. musculus	*Mus musculus*
mRNA	Messenger RNA
ms	Millisecond
NaCl	Sodium chloride
nm	Nanometre
NMR	Nuclear magnetic resonance
N-terminal	Amino-terminal
OD	Optical density
ONPG	Ortho-2-Nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
P. anserina	*Podospora anserina*
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
pmol	Picomole
psi	Pound-force per square inch
RD	Radicicol
RFP	Red fluorescent protein
RH	Right hand
rpm	revolutions per minute
s	Second
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH3	Src Homology 3 domain
SM	Striated muscle
S. pombe	*Schizosaccharomyces pombe*
TAE	Tris-acetate
TBS-T	Tris-buffered saline plus Tween
TEMED	Tetramethylethylenediamine
TPR	Tetratricopeptide repeat
ts	Temperature sensitive
U	Units
V	Volt
WT	Wild type
xg	centrifugal force
Y2H	Yeast two hybrid

Chapter 1: Introduction

1.1 Molecular chaperones and protein folding

Environmental conditions are in continuous change, causing stresses that can risk life. Free living microbes have mechanisms that reduce the detrimental effects of stress and maintain homeostasis. One important response to changing environment is the heat shock response, causing up-regulation of expression of a subset of protective proteins known as the heat shock proteins (Hsps) (Morano et al., 2012; Morimoto, 1998). Many of these are molecular chaperones whose general function, during normal conditions, is to assist in protein folding. However they also aid in the recovery of protein damage under stress. Chaperones are defined as proteins that help other proteins attain their final conformation more efficiently, but do not form a part of the final biologically-active state of these proteins. By performing "holdase" or "foldase" functions (Figure 1.1) they prevent protein-protein aggregation, assist folding of proteins which have been recently synthesised by the ribosome, assembling and folding immature proteins or, in the event that these cannot be folded properly, assist transit towards the pathways for protein degradation. Finally, chaperones are also involved in translocating proteins to other compartments so that the new folded proteins can perform their correct cellular function.

The heat shock response is highly conserved in evolution (Morano et al., 2012). There are several different families of Hsps and these often have different cellular localisations. They are classified into several structurally unrelated families on the basis of their molecular size in kDa, the major families being Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small HSPs. Of these Hsp families the Hsp90 family is of particular interest since it is an unusual protein chaperone. Unlike certain other well-characterised chaperones such as Hsp70, Hsp90 does not participate in the initial *de novo* folding of most proteins. Instead it is required for the

final activation step of almost-folded proteins. Hsp90 acts on these 'clients' when they are already substantially folded (often after Hsp70 and Hsp40 family proteins have acted on them), so as to enable the final structural changes whereby they attain their fully active conformation. Hsp90 clients include many important signalling and regulatory proteins such as the glucocorticoid receptor which depends on Hsp90 for steroid-dependent activation, and many protein kinases such as the tyrosine kinases v-src and c-src which need Hsp90 activation to achieve full maturation (Richter and Buchner, 2001).

Hsp90 is an abundant essential protein in eukaryotic cells (1-2% of total cytosolic protein even in the absence of cellular stress) (Borkovich et al., 1989). Hsp90 has received much recent interest ever since it was identified as a promising anticancer drug target, since Hsp90 is critical for the activation of many important oncoproteins, such as p53, v-src/c-src and Cdk4. The particular interest of Hsp90 as a drug target for cancer therapy comes from the fact that Hsp90 inhibition can inactivate simultaneously several Hsp90-dependent activities involved in multistep oncogenesis (Neckers and Workman, 2012). This provides an efficient way to simultaneously target several oncoproteins using only one drug, several oncogenic activities being targeted to the proteasome for degradation when cancer cells are treated with Hsp90 inhibitors (Connell *et al*., 2001). A number of Hsp90 inhibitors are now in clinical trials (Neckers and Workman, 2012)

Another aspect that has boosted interest in the Hsp90 field is the involvement of this chaperone in determining evolutionary change (Queitsch et al., 2002; Rutherford and Lindquist, 1998). Hsp90 activity is a key factor determining whether the genotypic variation that affects development pathways in nature is expressed phenotypically. Under non-stress conditions much of this variation is kept in silent since Hsp90 can buffer this genetic variation by assisting mutant client proteins achieve their wild-type conformation. However

if Hsp90 function is compromised during development, either by supra-optimal temperature or by Hsp90 inhibitors, the silent traits of these mutant client proteins are now expressed, leading to new genetic diversity. These new phenotypic traits can then be stably fixed in the population in response to evolutionary pressure, allowing a rapid emergence of adaptive changes in response to sudden environmental change.

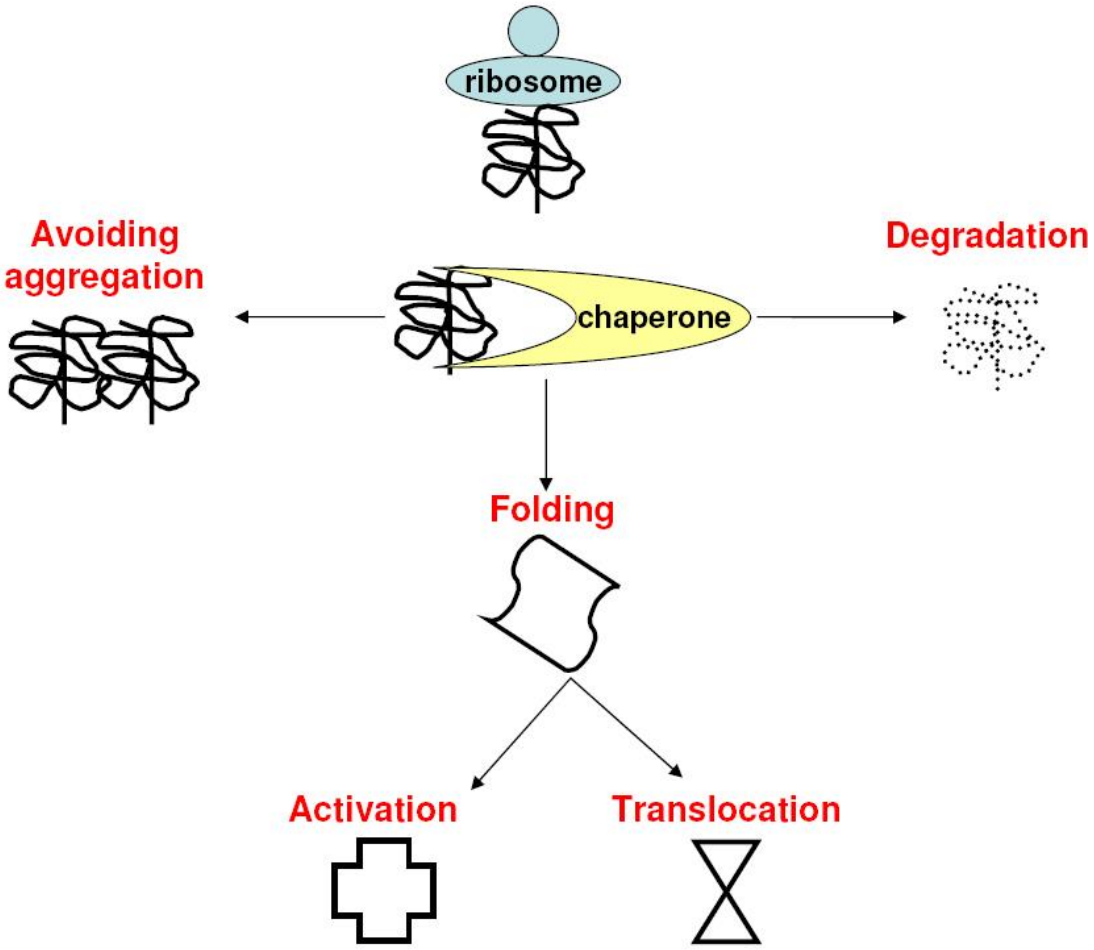


Figure 1.1 Proteins newly synthesised on the ribosome associate with molecular chaperones which assist several stages of protein maturation. These include avoiding protein aggregation, folding, activation and translocation to other cellular compartments or degradation. Hsp90 is mainly involved in the final activation of already largely-folded proteins.

1.2 The heat shock response

The heat shock response consists of activating a programme of heat shock gene expression to overcome stress. This mechanism is extremely well conserved and involves increasing Hsp expression as rapidly as possible. In eukaryotic organisms, this is directed by heat shock factor (Hsf), a stress-induced transcription factor. This Hsf binds to a characteristic DNA promoter element (the heat shock element HSE) consisting of an alternating repeats of the pentanucleotide sequence 5'-nGAAn-3' (Morano et al., 2012; Verghese et al., 2012). The interaction between the Hsf and the HSE is well conserved among eukaryotic organisms. However, there is wide variability in the number of HSF genes in different organisms. Yeast such as *S. cerevisiae* have just a single *HSF* gene (Hsf1), whereas up to four different Hsf isoforms can co-exist in plant, avian and mammalian cells. These multiple Hsf isoforms are involved not only in the cellular response to internal and external stimuli but also in other complex processes such as development and differentiation for reviews see: (Morano et al., 2012; Verghese et al., 2012).

There are important differences between the Hsfs of yeasts and higher eukaryotes (Sorger et al., 1987). In *S. cerevisiae* Hsf1 is constitutively trimerised, much of it being constitutively bound to HSEs in the absence of stress and under stress conditions. Under stress conditions, Hsf1 becomes hyperphosphorylated and functionally activated, leading to transcription of its target genes. Unlike the yeast form, the mammalian stress-inducible isoform Hsf1 is maintained in an inactive monomeric state. Heat or superoxide stress triggers the conversion of this monomer into a homotrimer with DNA-binding affinity, cysteine residues in the DNA-binding domain becoming oxidised and forming redox-sensitive disulfide bonds (Ahn and Thiele, 2003). This active homotrimer is then translocated to the nucleus where it binds

the HSEs at the promoter regions of *HSP* genes to initiate transcription (Morano et al., 2012; Verghese et al., 2012).

Another notable difference between the Hsf1s of yeast and metazoans is that the latter possess just a single trans-activation domain whereas yeast Hsf1 has two independent transcriptional activation domains. Due to their position the latter are named the amino-terminal transcriptional activation domain (NTA; residues 1 to 172) and carboxyl-terminal transcriptional activation domain (CTA; residues 584 to 833) (Nieto-Sotelo et al., 1990; Truman et al., 2007). It seems that these two HSF activator regions, NTA and CTA, perform different functions. The NTA is sufficient for transient increases in Hsf1 activity whereas the CTA mediates the sustained response. DNA microarrays showed that some chaperones have activation domain specificity. Expression of *HSP40* was dependent on the NTA whereas the CTA was needed for expression of *HSP82*, the yeast heat-inducible isoform of Hsp90 (Nieto-Sotelo et al., 1990).

1.3 The yeast *Saccharomyces cerevisiae* as a model organism for studying Hsp90

Hsp90 is highly conserved among eukaryotes and in most species Hsp90 family proteins are to be found in the cytosol, endoplasmic reticulum, mitochondria and chloroplasts. Some fungi are an exceptions, possessing only a cytosolic Hsp90 (Chen et al., 2006). Cytosolic Hsp90 exists as a single form in *D. melanogaster*; two (Hsp90α and Hsp90β) isoforms in humans and seven Hsp90 isoforms in *A. thalania*. In the budding yeast *S. cerevisiae* cytosolic Hsp90 is required for viability, and this is a good model organism in which to study cytosolic Hsp90 function in view of its ease of genetic manipulation, also the ability to switch Hsp90 genes by plasmid shuffle (Millson et al., 2007; Piper et al., 2003). *S. cerevisiae* has two 97% identical isoforms of cytosolic Hsp90; Hsp82 and Hsc82, these sharing 50-60%

sequence identity with the two isoforms of human cytosolic Hsp90; Hsp90α and Hsp90β. Expression of the latter human Hsp90s as sole Hsp90 in *S. cerevisiae* can provide the essential Hsp90 function in this yeast (Millson et al., 2007; Piper et al., 2003).

1.4 Structure of the Hsp90 chaperone

When unfolded, newly synthesised proteins emerge from the ribosome their initial folding is normally facilitated by chaperones of the Hsp40/Hsp70 family. Subsequently Hsp90 facilitates the final folding/activation of a number of these proteins both under normal and stressful conditions. Elucidating how Hsp90 acts on these varied substrates is one of the main challenges in the chaperone field. Structural studies of Hsp90 in complex with its clients and/or co-chaperones can give us a better understanding of this mechanism. Nuclear magnetic resonance (NMR) and X-ray crystallography have shown Hsp90 consists of a modular structure with two well conserved regions, a ~25 kDa N-terminal domain joined by a highly charged 'linker' region to a ~55 kDa C-terminal domain. It comprises three different domains: an N-terminal ATP binding domain, a middle domain thought to be the major site of client protein binding and a C-terminal domain, the site of constitutive dimerisation (Figure 1.2.a). The crystal structure of the full-length Hsp90 in complex with its co-chaperone p23/Sba1 ((Ali et al., 2006); Figure 1.2) reveals these domains within the compact dimeric state of Hsp90. However this very flexible chaperone is now thought to undergo a number of conformational changes as it fulfils its function, changes that are largely directed by ATP- and cochaperone binding (Figure 1.3).

1.4.1 The N-terminal Domain of Hsp90

The ~25 kDa N-terminal conserved domain of Hsp90 binds ADP/ATP and is also the site of interaction of the ATP mimetic Hsp90 inhibitors, geldanamycin (GA) and radicicol (RD) (Prodromou et al., 1997; Roe et al., 1999). The crystal structure of this N-terminal ATP-

binding site showed that it is structurally similar to GHKL family ATPases, the latter including type II topoisomerases such as DNA gyrase B, the MutL DNA mismatch repair protein and the bacterial histidine kinase CheA.

1.4.2 Hsp90's ATPase activity

Purified Hsp90 has an inherent ATPase activity, inhibited by the Hsp90 inhibitors GA and RD (Panaretou et al., 1998; Prodromou et al., 1997; Roe et al., 1999). Two key residues on the N-terminal domain were found to be important in the ATPase activity of yeast Hsp90. Aspartate D79 is required for ATP binding. When it is mutated from aspartic acid to asparagine ATP binding is disfavoured but the nucleotide binding pocket is not disordered (Panaretou et al., 1998). Glutamate E33 was found to be conserved between Hsp90 and DNA gyrase B, which suggested it is necessary for ATP hydrolysis. When these mutations are expressed in yeast as their sole form of Hsp90 the cells are unable to survive (Panaretou et al., 1998). This demonstrated that the *in vivo* function of Hsp90 requires its intrinsic ATPase activity.

1.4.3 The dimeric nature of Hsp90

The two subunits of the Hsp90 dimer exhibit a constitutive association at their C-terminal domains, yet an additional transient association of the N-terminal domains upon ATP binding (Meyer et al., 2003; Prodromou et al., 2000). This chaperone is therefore suggested to act as a ´molecular clamp´, in which ATP-binding by Hsp90 causing a conformational change that traps the client protein into a tight complex (Figure 1.3). With ATP hydrolysis the ´molecular clamp´ may then return to its "open" conformation releasing the now-activated client protein. The structure of full-length Hsp90 in complex with the co-chaperone p23/Sba1 and the ATP analogue AMP-PNP ((Ali et al., 2006); Figure 1.2) revealed how the co-chaperone p23/Sba1 is recruited to stabilise the ATP-bound complex the full Hsp90

architecture. This structure confirmed that Hsp90 functions as a dimer in which the N-terminal domains transiently associate, but it did not support the notion that the client protein was located inside the ´molecular clamp´ as the gap between the middle domains of the Hsp90 dimer is too small in the ATP bound conformation to enclose a client protein. Instead it indicated that Hsp90 substrates associate with a bipartite binding surface, between the N-terminal and middle domain, in a manner coupled to the chaperone ATPase cycle. This was also indicated by a structure obtained by cryo-electron microscopy of the kinase Cdk4 in complex with Hsp90 and co-chaperone Cdc37. In this the kinase substrate seems to be located on both the N-terminal and middle domains of Hsp90 (Vaughan et al., 2006).

1.4.4 The central highly charged region of Hsp90

The charged 'linker' region joins the N-terminal and middle domains of Hsp90. It is the least conserved section of the Hsp90 protein and is completely absent from bacterial HtpG. Deletion of much of this linker in the yeast Hsp82 showed does not have any effect on the ATPase activity of Hsp90 *in vitro* (Prodromou et al., 2000) and that it is not required for the essential function of the cytosolic chaperone *in vivo* (Louvion et al., 1996).

1.4.5 The middle (client Binding) domain of Hsp90

The structure of the middle region of yeast Hsp90 (residues ~ 270-550) revealed three different regions: a large αβα domain at the C-terminus which is connected to a smaller αβα domain by a helical coil segment (Meyer et al., 2003). The architecture of these segments is very similar to the GHKL ATPase family (Meyer et al., 2003).

1.4.6 The C-terminal domain of Hsp90

Besides being the region responsible for the constitutive dimerisation of Hsp90, the C-terminal region of cytosolic Hsp90 also ends in the conserved sequence motif -MEEVD. The latter is binding site for tetratricopeptide repeat (TPR) domain containing co-chaperones (Blatch and Lassle, 1999), some of which "bridge" Hsp70 and Hsp90 so as to assist the assembly of Hsp70-Hsp90 multichaperone complexes (Scheufler et al., 2000). Remarkably, neither this motif nor many TPR-containing Hsp90 co-chaperones (with the exception of Cns1 (Dolinski et al., 1998)) are necessary for *S. cerevisiae* viability (Louvion et al., 1996).

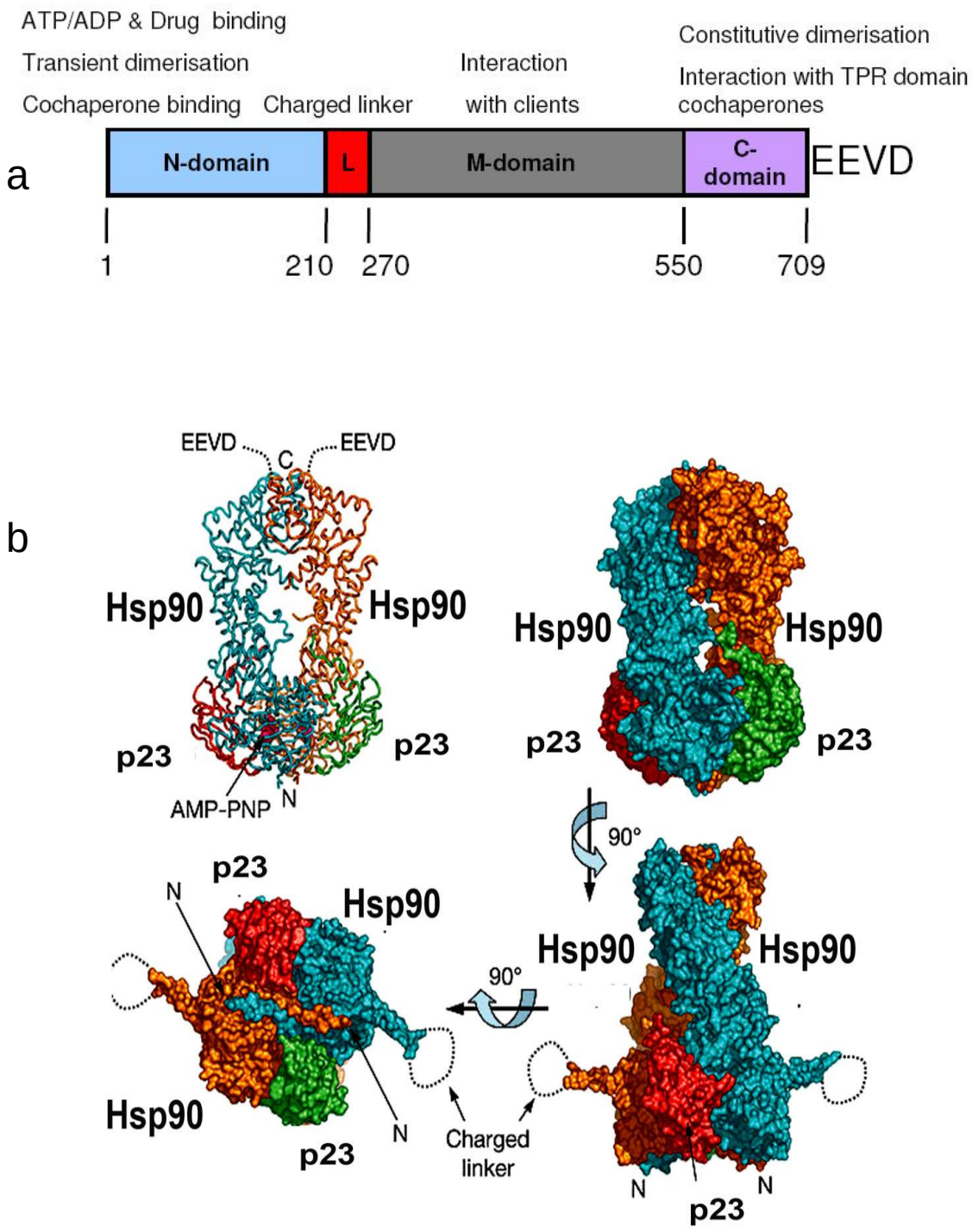


Figure 1.2. Structure of Hsp90 (a) Schematic representation of the amino acid domain structure of Hsp90 and their biochemical functions. L refers to the ´Charged linker´ region which serves as a flexible linker between the N and Middle domains. The EEVD motif is the TPR co-chaperone site binding. (b) Structure of the Hsp90 dimer with the co-chaperone p23/Sba1. Blue and orange represents each Hsp90 monomer that are associated via the C-terminus. Red and green represents p23/Sba1 that binds to the N-terminus, the pink point indicates the ATP analogue AMP-PNP (Ali et al., 2006).

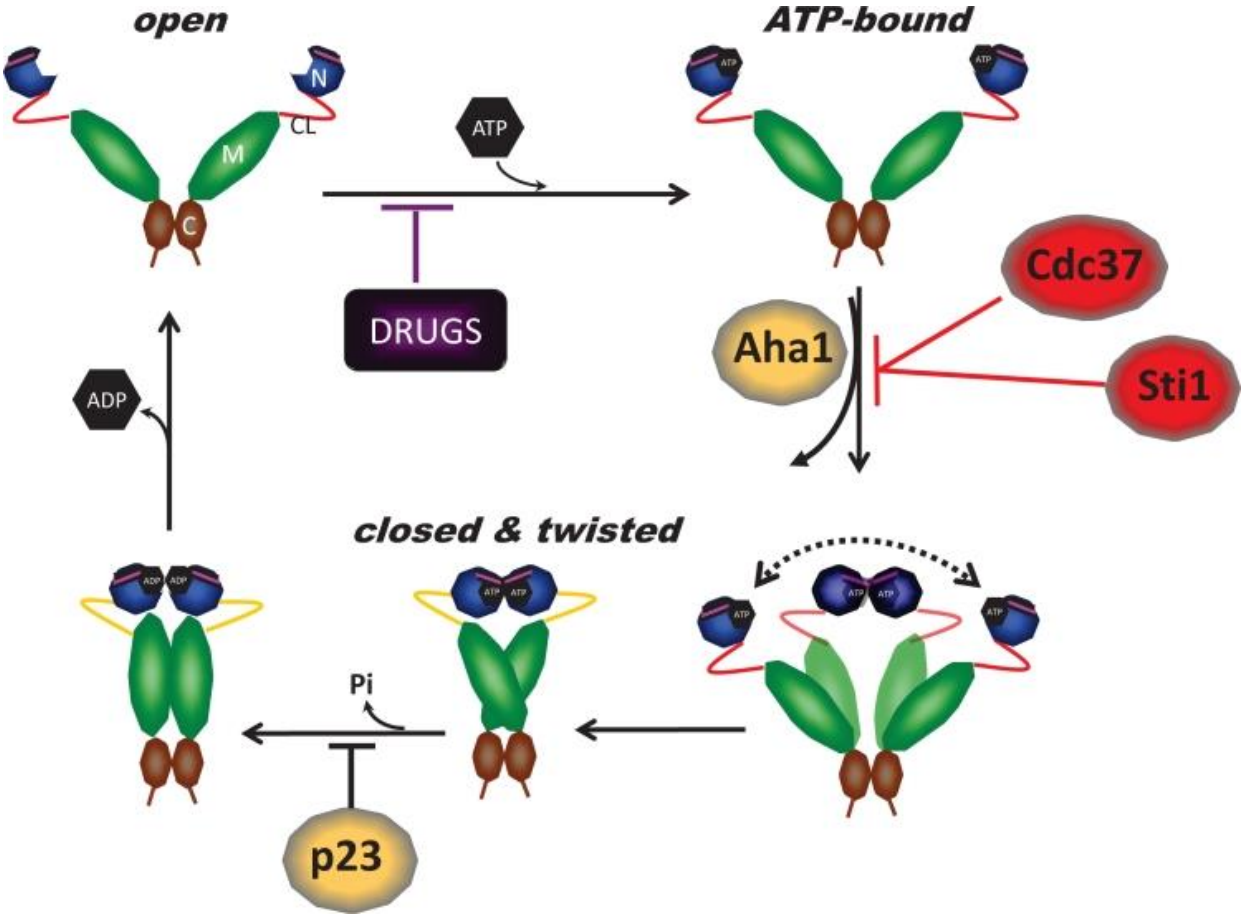


Figure 1.3. The ATP-driven molecular clamp mechanism of Hsp90 (Mollapour et al., 2011). Hsp90 is constitutively dimerised at its C-Terminus (brown). ATP binding at the N-terminal domain (blue) leads to a conformational change causing the N-terminal regions to dimerise transiently. This new Hsp90 conformation is facilitated by some cochaperones (e.g. Aha1) but inhibited by others (e.g. Cdc37, Sti). It causes a tighter association with the client protein (not shown) which is only relieved when Hsp90´s ATPase activity hydrolyses the ATP causing the 'clamp' to re-open and release of the activated client protein. Hsp90 inhibitors such as GA and RA are ATP mimetics and therefore inhibit this ATP cycle.

1.5 Client proteins of Hsp90

Genomic studies indicate that up to 10% of the proteome in eukaryotic cells may associate with Hsp90 (Franzosa et al., 2011; Millson et al., 2005; Zhao et al., 2005). Many of these associations are essential for protein functionality, these 'client proteins' achieving their biologically active state through Hsp90/co-chaperone association. The Hsp90 clientele identified so far comprises proteins that are all very different, structurally and functionally, which is presumably one reason why Hsp90 also needs a number of specific co-chaperones, chaperones and cofactors to selectively target its substrates. Examples of these clients proteins include telomerase, steroid receptors, tumour suppressor p53, Hsf1, oncogenic v-src/c-src, Wee1, Cdc2, Cdk4/6/9, nitric oxide synthase, actin, and tubulin. Some of the best studied Hsp90 clients are protein kinases, assisted by the kinase-specific co-chaperone Cdc37 (see below), and nuclear steroid hormone receptors the latter achieving their active state with the help of the chaperones Hsp70/Hsp40 and the co-chaperone Hop/Sti1.

Steroid hormone receptors like the glucocorticoid (GR) and progesterone receptors are some of the most studied Hsp90 clients. The steroid hormone receptor is localised in the cytosol in complex with Hsp70 and Hsp40, with the binding cleft in a conformation which does not allow access for hormone. This complex is then loaded onto the Hsp90 dimer by Hsp70 with the assistance of the co-chaperone Hop/Sti1. ATP dependent binding of p23/Sba1 to Hsp90, displaces Hsp70 and Hop, allowing immunophilin FKBP51/2 binding. The final complex leads to fully mature steroid hormone receptor which can then bind steroid and migrate to the nucleus (Figure 1.4).

Hsp90 needs the assistance of a variety of accessory "co-chaperone" proteins when activating specific proteins i*n vivo*. Depending on the client being studied, its function has been shown to need more than 12 of these co-chaperones, in addition to the multiple forms

of the Hsp70 chaperone and the Hsp70 co-chaperones. Identifying the mechanisms whereby these co-chaperones module the Hsp90 cycle to activate specific clients is a remarkable challenge in the Hsp90 field.

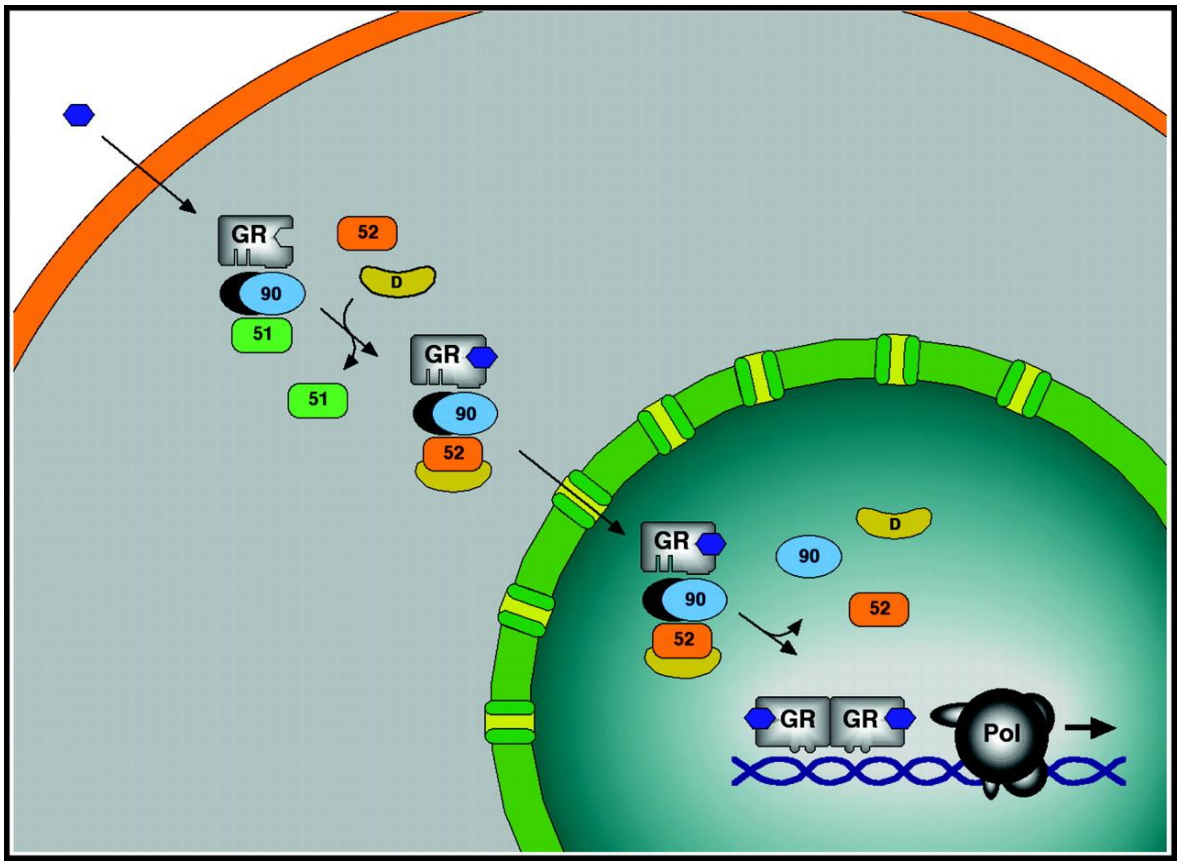


Figure 1.4. Activation of glucocorticoid receptor (GR) by hormone and Hsp90. Hormone binding to GR causes Hsp90 association and movement from the cytoplasm into the nucleus of the GR complexed with Hsp90 and the immunophilin FKBP51. Binding of hormone to GR causes a conformational change in the complex, which results in exchange of FKBP51 for FKBP52 (Pratt et al., 2004; Pratt and Toft, 2003). FKBP52 in turn binds the dynein (dyn) motor protein attached to the cytoskeleton and transports the GR complex into the nucleus. Once in the nucleus, the complex disassembles releasing the GR, which dimerizes and binds to DNA where it facilitates transcription of DNA (diagram taken from Davies et al. 2002).

1.6 Cochaperone accessory proteins of the Hsp90 system

1.6.1 TPR domain containing co-chaperones

Many Hsp90 co-chaperones have one or more tetratricopeptide (TPR) repeat domains (Das et al., 1998), a protein-protein association domain that – in these cochaperones – facilitates binding to Hsp70 and/or Hsp90 by association with the C-terminal motif M(I)EEVD of Hsp70 and Hsp90. The co-chaperone Sti1 (p60/Hop in mammalian cells) acts as a scaffold protein, recruiting Hsp90 to the Hsp70 chaperone machine by interacting with Hsp70 via its C-terminal IEEVD motif and with Hsp90 via its MEEVD motif. In this way it "bridges" Hsp70 and Hsp90 and assists the assembly of Hsp70-Hsp90 multichaperone complexes (Scheufler et al., 2000). TPR-domain co-chaperone binding also plays an important role in the regulation of the ATPase activity of Hsp90. Sti inhibits the ATPase activity of Hsp90 by preventing access to the ATP binding pocket in the N-terminal domain (Prodromou et al., 1999). (Prodromou *et al*., 1999) but it has no effect on the ATPase activity of Hsp70 (Johnson et al., 1998).

Other TPR domain co-chaperones include CHIP, which regulates the degradation of client proteins (Zhang et al., 2005), the immunophilin FKBP52 which assists in nuclear import of the GR (Pratt et al., 2004; Pratt and Toft, 2003) (Figure 1.4), Tom70 which mediates protein import into mitochondria (Young et al., 2003) and Tah1 (Millson et al., 2008) which is involved in snoRNP and RNA polymerase assembly (Back et al., 2013). UNC45 is a TPR-domain containing co-chaperone that, in metazoans, assists in the folding and assembly of myosins (Barral et al., 2002) although the fungal homologues of this UNC45 (Cro1, She4) lack the TPR domain.

1.6.2 Cdc37 – a non-TPR containing co-chaperone

One of the most studied Hsp90 co-chaperones that lacks a TPR domain is Cdc37 (p50 in mammalian cells). Maturation and stabilization of nascent protein kinase molecules often requires an initial association with Cdc37(p50), these maturing protein kinase molecules then being targeting on to Hsp90 (Mandal et al., 2007; Pearl, 2005). While Cdc37 fulfils an essential function in diverse species from yeast to man, this important molecular chaperone would appear to be lacking in certain eukaryotic phyla (Johnson and Brown, 2009). Notably Cdc37 seems to be absent in the Apicomplexan phylum of protozoan pathogens, possibly a reflection of the protein kinases in these species differing, both structurally and mechanistically, from the protein kinases of their hosts (Talevich et al., 2011).

Since its original discovery of as a component of the v-Src-Hsp90 complex (Brugge et al., 1981), Cdc37(p50) has been found associated with a wide spectrum of protein kinases. A few non-kinase proteins have also been found to bind Cdc37 (Krogan et al., 2006; Millson et al., 2004; Rao et al., 2001), though the precise function of Cdc37 in the biology of these non-kinase clients is still poorly understood. While Cdc37 interaction serves to stabilise many newly-synthesised protein kinases (Mandal et al., 2007; Pearl, 2005), we remain largely ignorant of how such nascent protein kinases interact with Cdc37 at the structural level; especially how the N-terminus of Cdc37– the Cdc37 region highly conserved in evolution – might assist presentation protein kinases to the Hsp90 chaperone machine. A crystal structure has though revealed how the middle region of Cdc37 interacts with the N-terminal domain of Hsp90, thereby preventing ATP-driven N-terminal dimerisation and transiently arresting the Hsp90 chaperone cycle but allowing loading of the client protein kinase (Roe et al., 2004; Siligardi et al., 2002).

Important for Cdc37 function is a sequential casein kinase 2 (CK2)-catalysed phosphorylation, then Ppt1(PP5)-catalysed dephosphorylation, of evolutionarily conserved serines at the conserved N-terminal region of Cdc37 (Bandhakavi et al., 2003; Miyata and Nishida, 2004; Shao et al., 2003; Vaughan et al., 2008). Yeast Cdc37 is phosphorylated by CK2 on both Ser14 and Ser17, whereas the mammalian Cdc37 (p50) only Ser13 (equivalent to Ser14 in the yeast Cdc37) is modified in this manner (this p50 having a glutamic acid, a possible phosphomimetic residue, at the position equivalent to Ser17 in the yeast Cdc37). A yeast mutant with a nonphosphorylatable S14A mutant Cdc37 (*cdc37(S14A)*) is temperature sensitive, exhibiting poor growth even at moderate temperatures (Bandhakavi et al., 2003). It has ~70% of its kinome destabilized, revealing that S14 phosphorylation of Cdc37 is important for protecting nascent kinase chains against degradation and maintaining appropriate *in vivo* levels of protein kinases (Mandal et al., 2007). Our laboratory and others have shown that this Ser14 phosphorylation event is essential for the functionality of several stress-responsive, mitogen-activated protein kinase (MAPK) pathways in yeast, the *cdc37(S14A)* mutation causing loss of Cdc37 interaction with key signaling components of these pathways, notably the kinases Ste11, Kss1, Hog1 and Slt2 (Abbas-Terki et al., 2000; Hawle et al., 2007; Yang et al., 2006).

Another Hsp90 co-chaperone that lacks a TPR domain is Sba1 (p23 in mammalian cells). Like Sti1 this also has an inhibitory effect on the ATPase activity of Hsp90 (Siligardi et al., 2004) but whereas Sti1 binds Hsp90 in the absence of ATP, Sba1 binds Hsp90 only in the presence of ATP (Fang et al., 1998). Sba1 interacts with the closed conformation of Hsp90 when the two N-terminal domains are dimerised and the ATP and the client protein bound. At this position Sba1 reinforces the ATP-bound conformation of Hsp90 and is thought to stabilise the complex in which the client protein achieves its activatory conformational change, allowing release of this client protein when ATP is hydrolysed (Ali et al., 2006).

This ATP hydrolysis is also stimulated by another non-TPR co-chaperone binding to the middle domain of Hsp90, Aha1 (Meyer et al., 2004; Panaretou et al., 2002).

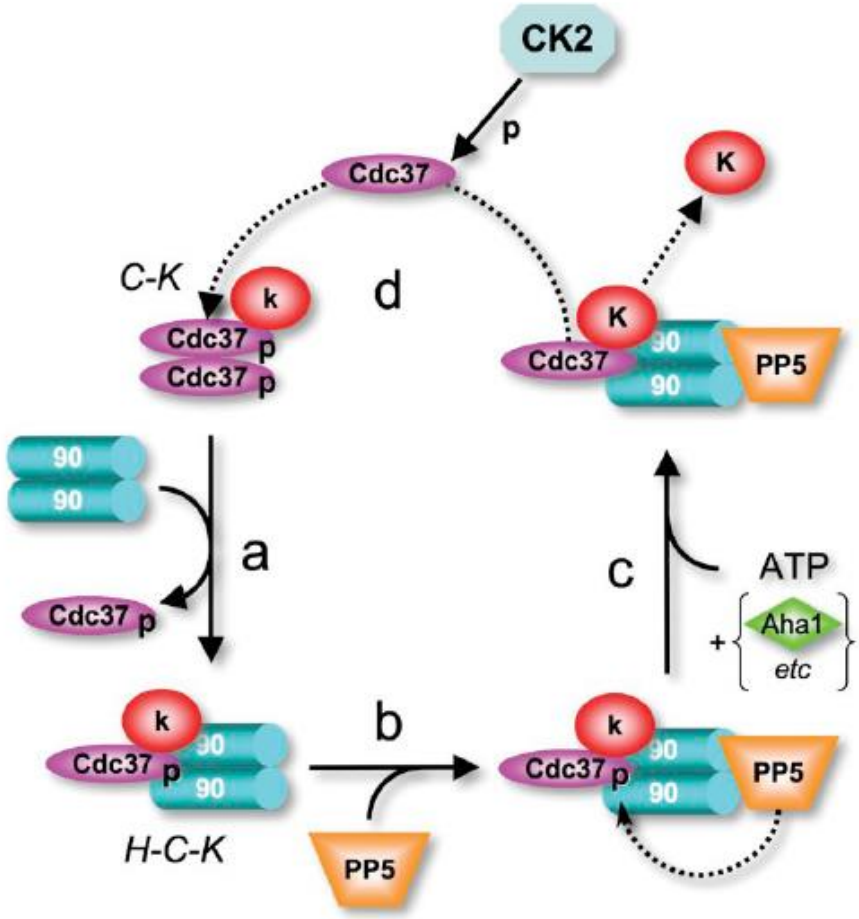


Figure 1.5. Activation of protein kinases by the Hsp90-Cdc37 cycle. Maturation of many protein kinases (K) requires phosphorylation/dephosphorylation of the kinase-specific chaperone Cdc37, then transfer of the nascent kinase to Hsp90. (a) Cdc37, phosphorylated by CK2, targets the inactive kinase and Hsp90 dimer forming the H-C-K complex. (b) PP5 (Ppt1 in yeast) phosphatase binds to Hsp90 and dephosphorylates Cdc37, (c) which subsequently dissociates from the Hsp90 complex. ATP binding and hydrolysis by Hsp90 together with additional co-chaperones leads to activation and release of the kinase. (d) Free Cdc37 is re-phosphorylated by CK2 at Ser14 (yeast Cdc37) or Ser13 (human Cdc37), thus "priming" for another Hsp90-kinase cycle activation (Reproduced from (Vaughan et al., 2008)).

1.7 Outline of the studies in this thesis

Most Cdc37-nascent protein kinase associations are extremely transient in the environment of the living cell. Therefore they are generally not detectable by the yeast two hybrid system. Chapter 3 analyses a Cdc37-kinase interaction that was detectable by two hybrid screening, the Cdc37 interaction with the most atypical protein kinase of yeast, cyclin-dependent kinase (Cdk)-activating kinase (Cak1) (Figure 1.6).

In **Chapter 3** it is shown that Cdc37 and Cak1 form a complex that is substantially unaffected by Cdc37 phosphorylation status, a complex that was sufficiently stable as to be isolatable by gel filtration. Formation of this Cdc37/Cak1 complex was found to be associated with a change to the phosphorylation status of Cak1. Mass spectrometry identified two new sites of Cak1 phosphorylation, Thr27 and Ser172. In **Chapter 4** yeast strains were constructed in which the sole, essential Cak1 has either a nonphosphorylatable or a phosphomimetic amino acid at these sites, also the Ser205 identified as phosphoryated in an earlier study. These were then analysed for altered cell cycle progression and stress-sensitivity.

Chapter 5 analyses Cdc37 stability in yeast, in particular how the stability of this normally stable chaperone is dramatically affected by molybdate treatment and oxidative stress.

The studies in this thesis provide the first demonstration that: (i) Cdc37 and Cak1 form a stable complex (Chapter 3); (ii) yeast Cak1 is regulated by phosphorylation (Chapter 4); and (iii) Cdc37 is a determinant of oxidative stress in yeast, being degraded with this stress and a molybdate treatment that also causes a discrete fragmentation of Hsp90 (Chapter 5).

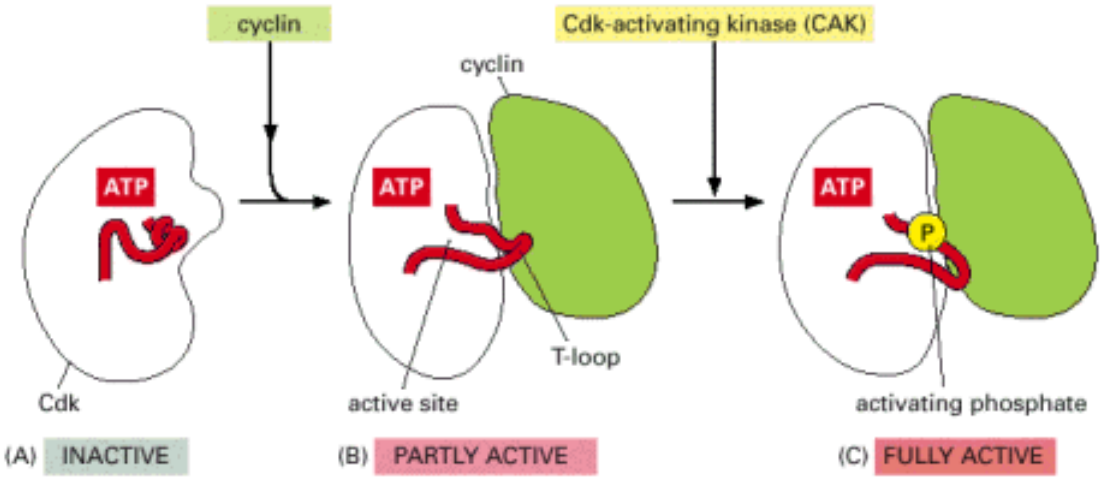


Figure 1.6 Cak1's essential role in yeast is to catalyse activatory Thr169 phosphorylation of Cdc28 cyclin dependent kinase (Cdk); its effects being counteracted by Swe1/Wee1 tyrosine kinase phosphorylation of Cdk on Tyr18. Cak1 also performs nonessential functions, phosphorylating Cdks that regulate transcription by RNA polymerase II (Kin28, Bur1, Ctk1) and components of the meiotic apparatus.

Chapter 2: Materials and Methods

2.1 Yeast growth media and culture conditions

Yeast cultures were either grown in rich media (YPD), nitrogen base media or synthetic defined minimal media (SD) with the appropriate amino acid dropout (recipes as below (all % values are w/v) with plates of each media made by addition of Bacto-agar (2%) prior to autoclaving at a temperature of 120°C (15 psi) for 15 min):-

YPD: D-glucose (2%) (Thermo Fisher Scientific Inc., Loughborough, UK); Bacto-peptone (2%); Bacto-yeast extract (1%).

SD: 2% D-glucose, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic amino acids where required: adenine (20mg/l), L-histidine (20 mg/l), L-leucine (30 mg/l), L-lysine (30 mg/l), L-tryptophan (20 mg/l), uracil (20 mg/l).

Yeast nitrogen base without amino acids and bases: 2% of chosen carbohydrate, 0.67% yeast nitrogen base (without amino acids) plus one or more of the following auxotrophic amino acids where required: adenine (20 mg/l), L-histidine (20 mg/l), L-leucine (30 mg/l), L-lysine (30 mg/l), L-tryptophan (20 mg/l), uracil (20 mg/l).

Dropout media: SD base (26.7 g) or nitrogen base (6.7 g) and the appropriate amount of complete synthetic media supplement lacking the required amino acids were added per litre of required media. All liquid and agar media adjusted to pH 6.5.

Growth media and agar were supplied by (BD Ltd., Oxford, UK); SD base and supplements were supplied by (Melford Ltd, Ipswich, UK; Formedium Ltd, Hunstanton, UK; Sigma-Aldrich, Gillingham,UK).

All yeast transformants containing the gene of interest in the pYES2.1/V5-His-TOPO® plasmid were grown either in YPD or -URA 2% Glucose. For induction of the expression of the protein under the *GAL1* promoter, cells were grown in -URA 2% Galactose.

2.2 Yeast strains used in this study

The yeast strains used in this study are listed in (Table 2.1) they were maintained as frozen stocks in YPD + 15% (v/v) glycerol at –80°C.

To generate strains DH211-5 CAK1-myc (Table 2.1) the *CAK1* coding sequence in DH211-5 (Hawle et al., 2007) was tagged at its C-terminus with the *myc* epitope, using a cassette amplified using a *13myc-TRP1* vector (Longtine et al., 1998) as template and primers CAK1tag-R1

(TATGGGTGTGACCGCTTGCAACAAGAATTTACCGTACATGAAGAATTCGAGCTCGTTTAAAC) and CAK1tag-F2

(GAAAGATGGAGCTGTCAAAGAATCTTGCAAGAATTA**GAA**AAG**CCA**CGGATCCCCGGGTTAATTAA) (Homology to the cassette underlined). Correct integration in Trp+ trasnformants of DH211-5 was confirmed by colony PCR (Section 2.4.3), using primers CAK1-chkR (CCTTCTAACATGTCTGCGCACGGG) and CAK1-chkF (GCGATTTCAGACTGATTTGCTC), these annealing at +1400 and +790 relative to the first base of the start codon respectively.

For inserting mutant *cak1* genes into SY80 (Table 2.1), the relevant TOPO clone (section 2.4.6) was first used as a template in a PCR reaction with the primers pHscCak1F and

pHscCak1R (Table 2.2; homology to pHSCprom in capitals and to *CAK1* in small case). SY80 cells were then transformed to leucine prototropy with this PCR product and linearised pHSCprom (Panaretou et al., 1999).

Table 2.1 Yeast strains

Strain	Genotype	Ref.
W303-1A	MAT-a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Cross and Levine, 1998)
82a	W303-1A *hsc82::LEU2 hsp82::LEU2 HSP82::HIS3*	(Nathan and Lindquist, 1995)
hsp90-G81S	W303-1A *hsc82::LEU2 hsp82::LEU2 hsp82,G81S::HIS3*	(Nathan and Lindquist, 1995)
SY80	W303-1A *cak1::HIS3 (CAK1-URA3)*	(Cross and Levine, 1998)
YDH6	W303-1A *cka1::HIS3 cka2::TRP1* (pRS315-*CKA2*)	(Bandhakavi et al., 2003)
YDH13	W303-1A *cka1::HIS3 cka2::TRP1* (pRS315- *cka2* ^{ts}*)*	(Bandhakavi et al., 2003)
TM141	MAT-a *leu2 ura3 trp1 his3*	(de Nadal et al., 2003)
DH211 CAK1-myc*	TM141 *cdc37::HIS3* (Ycplac111 *CDC37-HA*) *CAK1-myc-TRP1*	This study
DH212 CAK1-myc*	TM141 *cdc37::HIS3* (Ycplac111 *cdc37-S14A-HA*) *CAK1-myc-TRP1*	This study
DH213 CAK1-myc*	TM141 *cdc37::HIS3* (Ycplac111 *cdc37-S14E-HA*) *CAK1-myc-TRP1*	This study
DH214 CAK1-myc*	TM141 *cdc37::HIS3* (Ycplac111 *cdc37-S17A-HA*) *CAK1-myc-TRP1*	This study
DH215 CAK1-myc*	TM141 *cdc37::HIS3* (Ycplac111 *cdc37-S17E-HA*) *CAK1-myc-TRP1*	This study
XX201	TM141 *cdc37::HIS3* (YCplac33 *CDC37-GFP*)	(Yang et al., 2006)
XX300	Hsp82-G81S (YEplac195 *CDC37*)	(Yang et al., 2006)
XX301	Hsp82-G81S (YEplac195 N-term *GFP-CDC37*)	P. Hawle thesis 2008
XX302	Hsp82-G81S (YEplac195 N-term *GST-CDC37*)	P. Hawle thesis 2008
PJ694-a	MAT-a *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::Gal1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James et al., 1996)
PJ694-α	MAT-α *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::Gal1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James et al., 1996)
PH600	PJ694-α (pBDC *CDC37-BD*)	P. Hawle thesis 2008
PH601	PJ694-α (pBDC *cdc37(S14A)-BD*)	P. Hawle thesis 2008

PH602	TM141 *cdc37::HIS3* (pBDC *CDC37-BD*)	P. Hawle thesis 2008
PH603	TM141 *cdc37::HIS3* (pBDC *cdc37(S14A)-BD*)	P. Hawle thesis 2008
MGY140	MATα *ura3-1 trp1-289 his3 leu2 lys2Δ0 mob1::kanR cdc28::LEU2 pep4::LYS2* (*pURA3-MOB1 CDC28*).	(Geymonat et al., 2007)
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
BY4741*ppt1*	BY4741 *ppt1Δ kanMX4*	Euroscarf
FY1679-28c	*MAT*-a *ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63*	Euroscarf
ctt1	FY1679-28c *ctt1*Δ*kanMX4*	Euroscarf
PP30-HSP82	MAT-a *trp1-289, leu2-3,112, his3-200, ura3-52, ade2-101^{oc}, lys2-801^{am}, hsc82::kanMX4, hsp82::kanMX4* (pHSCprom-*HSP82* [a] (*LEU2*)) **	(Millson et al., 2007)
PP30-huHSP90α	MAT-a *trp1-289, leu2-3,112, his3-200, ura3-52, ade2-101^{oc}, lys2-801^{am}, hsc82::kanMX4, hsp82::kanMX4* (pHSCprom- *huHSP90α* [a] (*LEU2*)) **	(Millson et al., 2007)

*Strains DH211-5 from an earlier study (Hawle et al., 2007) here modified to express a myc-epitope tagged Cak1.

** The sole Hsp90 gene in this strain, expressed under HSC82 promoter control and borne on the *LEU2* plasmid pHSCprom.

Table 2.2 Primers used in CAK1 gene cloning and CAK1 gene mutagenesis

Primer name	Sequence
Y2HCak1F	AATTCCAGCTGACCACCATGAAACTGGATAGTATAGAC
Y2HCak1R	GATCCCCGGGAATTGCCATGTTATGGCTTTTCTAATTCTTG
pHscCak1F	ACAGAACCAATAGAAAAATAGAATCATTCTGAAATATGAAACTGGATAGTATAGAC
pHscCak1R	CATAAATCATAAGAAATTCGCCCGGAATTAGCTTGGTTATGGCTTTTCTAATTCTTG
pHscCak6His Tag	ACAGAACCAATAGAAAAATAGAATCATTCTGAAATATGCATCATCATCATCATCATAA ACTGGATAGTATAGAC
Cak1T27Af	ACTGCTAGGATTTATAGGTCGGATGCATATGCCATTAAATGTCTAGCACTA
Cak1T27Ar	TAGTGCTAGACATTTAATGGCATATGCATCCGACCTATAAATCCTAGCAGT
Cak1T27Df	ACTGCTAGGATTTATAGGTCGGATGACTATGCCATTAAATGTCTAGCACTA
Cak1T27Dr	TAGTGCTAGACATTTAATGGCATAGTCATCCGACCTATAAATCCTAGCAGT
Cak1S172Af	CTAACAAACAATACCAGCACCGTAGCCCCAAAGTTGTACATAATTGATTTT
Cak1S172Ar	AAAATCAATTATGTACAACTTTGGGGCTACGGTGCTGGTATTGTTTGTTAG
Cak1S172Ef	CTAACAAACAATACCAGCACCGTAGACCCAAAGTTGTACATAATTGATTTT
Cak1S172Er	AAAATCAATTATGTACAACTTTGGGTCTACGGTGCTGGTATTGTTTGTTAG
Cak1S205Af	CCCATGGATAGCAAGGTGACGGATATAGCCACAGGAATTTACAAGGCCCCAGAAGTG
Cak1S205Ar	CACTTCTGGGGCCTTGTAAATTCCTGTGGCTATATCCGTCACCTTGCTATCCATGGG
Cak1S205Ef	CCCATGGATAGCAAGGTGACGGATATAGAGACAGGAATTTACAAGGCCCCAGAAGTG
Cak1s205Er	CACTTCTGGGGCCTTGTAAATTCCTGTCTCTATATCCGTCACCTTGCTATCCATGGG
Cak1Y221Ff	CCAGAAGTGCTTTTTGGAGTAAAATGCTTTGATGGTGGCGTGGACGTGTGGTCGTTG
Cak1Y221Fr	CAACGACCACACGTCCACGCCACCATCAAAGCATTTTACTCCAAAAAGCACTTCTGG

2.3 Yeast procedures

2.3.1 Yeast transformation

Transformation of *S. cerevisiae* strains with DNA was performed by making the yeast cells competent using lithium acetate (LiAc) and transforming with polyethylene glycol (PEG) and single-stranded DNA (Gietz *et al*., 1995). The yeast strain required for transformation was grown in the appropriate media from an OD_{600} of around 0.2 to an OD_{600} of around 0.8 at 30°C with agitation. The cells were harvested by centrifugation at 3,000 xg for 5 min and pellets were washed twice in 20 ml sterile dH_2O. The cell pellet was transferred to a sterile 1.5 ml microcentrifuge tube, centrifuged for 15 s at 3,000 xg, and excess water removed. The pellet was resuspended in 100 mM LiAc (1 ml) and incubated without agitation at 30°C for 30 min. After this period, the cells were again pelleted for 15 s at 3,000 xg and LiAc removed. The pellet was resuspended in 100 mM LiAc (500 µl) and for one transformation, a 50 µl aliquot of the cells was dispensed into a sterile 1.5 ml microcentrifuge tube. Excess LiAc was removed by centrifuging for 15 s at 3,000 xg. To each transformation, 240 µl PEG (50% w/v PEG 3350 Sigma-Aldrich Company Ltd., Dorset, UK), 30 µl 1 M LiAc, 25 µl single-stranded DNA (2 mg/ml), and 0.1-10 µg plasmid made up to 50 µl with sterile dH_2O was added. Each transformation reaction was mixed thoroughly by vortexing for 1 min before incubating at 30°C for 30 min followed by heat-shock at 42°C for 20 min both without agitation. Cells were pelleted at 3,000 xg for 15 s and the PEG supernatant was removed. Cell pellets were washed with 1 ml sterile dH_2O and resuspended in 500 µl sterile dH_2O. From each transformation reaction, a 100 µl aliquot was spread on the appropriate selection media agar plate. Transformants usually appeared after incubation for 3-4 days at 30°C.

2.3.2 Temperature growth assays

From an overnight culture of strains to be assayed, 100 µl of each was diluted down into 10 ml of the appropriate media in a sterile 50 ml centifuge tube. 150 µl of each diluted culture was aliquoted into rows of 12 adjacent wells of a 96-well V bottom plates. The plate was sealed and placed on a gradient PCR machine on a continuous cycle where wells 1-12 were on a gradient of temperature ranging from 30°C to 45°C. Plates were left for 24 h and the tolerance of the cultures to temperature was assessed by measuring cell density on a Multiskan Ascent microplate photometer (Thermo Fisher Scientific Inc.).

2.3.3 Plate growth assays

Plate growth assays were carried out with 10-fold serial dilutions of yeast strains in relevant media in 96-well flat bottom plates. Up to 6 dilutions of each strain were spotted onto relevant agar plates which were incubated from 2-5 days at the temperature required. Analysis of the rescue of osmosensitivity by Cdc37 constructs; also Hsp90 inhibitor sensitivity, were as previously described (Vaughan et al., 2008; Yang et al., 2006).

2.3.4 Cak1 and Cdc37 overexpression in yeast.

GAL1 promoter-driven overexpression of a N-terminally hexahistidine (His_6) tagged Cak1 used two vector systems; (i) His_6-CAK1 inserted into the *URA3* vector pYES2 (pYES2-His_6-CAK1) (for data in Fig 2d); (ii); His_6-TEV-Cak1 in the *HIS3/CDC28* vector pMH940 (Geymonat et al., 2007) (for data in Fig 3.5). For the latter, strain MGY140 was modified for dual *GAL1* promoter-driven overexpressions of both His_6-Cak1 and a Cdc37(S14A)-precission-Streptag fusion protein (Streptag introduced at level of PCR primer; see below). The His_6-TEV-Cak1 gene was amplified using primers: 919-CAK1F (***CAC****GATTACGATATCCCAACGACC*<u>*GAAAACCTGTATTTTCAG*</u>CTGGATAGTATAGACATTACACACT) and 919-CAK1R

(*TCTTAGCTAGCCGCGGTACCAAGCTTACTCGAGTCGACCCATGGAG*TTATGGCTTT TCTAATTCTTGCAAGA). Cdc37(S14A)-Streptag fusion expression used a modified form if the *TRP1/MOB1* vector pMH903 (Geymonat et al., 2007) in which an Nde1 site had been introduced at the start codon of the GST gene (TTCATG➔CATATG). By *Nde1* cleavage of this vector (pMH903-*Nde1*), homologous recombination with PCR-generated gene sequences can give vectors for *GAL1* promoter-driven overexpression of protein fusions with a C-terminal tag (in this instance it used a Cdc37 S14A)-precission-Streptag fusion gene amplified from strain DH212 DNA using primers: Cdc37F: (ttaatatacctctatactttaacgtcaaggagaaaaaaccccgGATCTcaggaaacagtaCATatggccattgattactctaa gtg) and Cdc37StrpR: (gaggtcgacggtatcgataagcttgatatcgaattctttttcgaactgcgggtggctccagtcaacagtgtcggcagtatgtttgac)

2.4 Molecular techniques

2.4.1 Amplification and modification of DNA by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) allows manipulations and amplification of template DNA. A reaction mix was prepared where the components of the reaction, excluding the template, were added together in a total volume of 50 µl. The mix contained 5 µl of 10x Expand™ high fidelity (HF) buffer with 15 mM $MgCl_2$ (Roche Diagnostics Ltd., West Sussex, UK) (20 mM Tris-HCl, pH 7.5; 100 mM KCl; 1 mM dithiothreitol DTT; 0.1 mM EDTA; 0.5% v/v Tween® 20; 0.5% v/v Nonident® p40; 50% v/v glycerol), 5 µl dNTP mix (dNTP mix is an aqueous solution containing dATP, dCTP, dTTP, dGTP, each at a final concentration of 2 mM (Fermentas, York, UK)), 0.5 µl of each primer (100 pmol), and 1 µl of 3.5 U Expand™ HF *Taq* DNA polymerase (Roche Diagnostics Ltd.). The relevant amount of template DNA was then added to the reaction, which was then made to a total of 50 µl using sterile dH_2O. PCR conditions varied depending on the DNA template being amplified

and the primers used but (Table 2.3) shows the general conditions as recommended by the manufacturer (Roche Diagnostics Ltd.). Usually, a small aliquot of the PCR was run on an agarose gel to see if a product of the required size had been produced

Table 2.3 General PCR parameters.

Number of cycles	Conditions
1x	Denature template 2 min at 95°C
30-35x	Denaturation at 95°C for 30 s Annealing usually at 45-65°C * for 30 s Elongation at 72 or 68°C ** for 1 min/kb
1x	Final elongation at 72°C for 10 min

*** The annealing temperature was determined for each primer pair using the TM calculator function of pDraw32 (ACALONE Software; http://www.acaclone.com/).**

**** Elongation temperature depends on the length of amplification product: 72**°C **is used for amplification up to 3.0 kb; 68**°C **is used for amplification >3.0 kb to prolong the activity of the polymerase**.

2.4.2 Site-directed mutagenesis

This PCR allows the site-specific mutation of nucleotide residues in a wild-type sequence of DNA. A 50 μl reaction mix was prepared as for a general PCR (Section 2.4.1) but using *Pfu* DNA polymerase, 10x reaction buffer with $MgSO_4$ in place of the Expand™ HF buffer (Promega Ltd., Southampton, UK) (200 mM Tris-HCl, pH 8.8; 100 mM KCl; 100 mM $(NH_4)_2SO_4$; 20 mM $MgSO_4$; 1% Triton® X-100; 1 mg/ml nuclease-free bovine serum albumin BSA). PCR was as in (Table 2.3) except the main number of cycles was 15-20 instead of 30-35 and the elongation step was always at 68°C. These changes are due to plasmid size being very large so with elongations ranging between 8-25 min, the activity of the polymerase needs to be prolonged. After PCR, reactions were treated with 20 U *Dpn*I (New England Biolabs) for 1 h at 37°C. *Dpn*I cuts at 5'-Gm^6ATC-3' and is specific for methylated DNA. Generally, DNA produced by *E. coli* (i.e. the original plasmid template) is *dam* methylated and therefore susceptible to *Dpn*I, while the PCR produced mutant DNA is unmethylated. This difference allows for an easy enrichment of intact mutant plasmid. After digestion the reaction was transformed into chemically competent (see Section 2.5.3). *E. coli* cells, selecting for the intact mutant plasmid. The resultant colonies were then screened for presence of the mutation by DNA sequencing (Cogenics Inc., Essex, UK).

2.4.3 Colony PCR

Colony PCR was used to screen for correct transformants. For yeast colony PCR, yeast cells had to be lysed to obtain crude DNA. A small amount of each colony was added to a 1.5 ml microcentrifuge. The tube was then heated on the microwave for 90 seconds to lyse the cells. The PCR mix was then added as described in section 2.4.1. For bacterial colony PCR, a little bit of each colony was added directly to the PCR mix since the cells will easily lyse at 95°C during the PCR cycle.

2.4.4 Isolation and purification of DNA fragments from agarose gels

DNA fragments were extracted and purified from an agarose gel using the QIAquick™ gel extraction kit following the manufacturer's protocol (Qiagen Ltd., West Sussex, UK). The S.N.A.P.™ DNA clean-up system (Invitrogen Ltd.) was used to isolate DNA fragments from agarose gel slices when they were required for TOPO cloning. This is due to the protocol being gentler than the QIAquick™ gel extraction kit (see Section 2.4.4) meaning a lower chance of the 3' A-overhangs being sheared away from the DNA fragments which would affect the efficiency of the TOPO® cloning. The manufacturer's protocol was followed (Invitrogen Ltd.).

2.4.5 Ligation of DNA fragments to plasmid vectors

DNA fragments with compatible ends to cut vectors were ligated together as described in Maniatis *et al*. (1989) using T4 DNA ligase (New England Biolabs) with the supplied 10x buffer at a 1x working concentration (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 1 mM ATP; 10 mM DTT; 25 μg/ml BSA). The DNA was used at concentrations between 0.01 and 0.1 μg using a stoichiometric ratio of approximately 3 DNA fragments to 1 of cut vector. The reaction was incubated overnight at 16°C, the optimum working temperature for T4 DNA ligase.

2.4.6 TOPO® XL PCR cloning and site-directed mutagenesis

When required, PCR products would be cloned into the pCR®-XL-TOPO® vector using the TOPO® XL PCR cloning kit (Invitrogen Ltd., Paisley, UK). During the PCR reaction, Taq polymerase adds a single deoxyadenosine to the 3` ends of PCR products, which tends to degrade away over time. The kit uses this overhang to ligate fresh PCR products into pCR®-XL-TOPO® as this vector is provided linearised, with 3` deoxythymidine overhangs, to which topoismerase has been bound to allow quick and efficient ligation of PCR products

(Shuman, 1994). A 50 µl PCR was set-up and run (See Section 2.4.1), but with a final extension at 72°C for 30 min. This allows extra time to make sure the nontemplate-dependent terminal transferase activity of Taq polymerase to add the 3' A-overhang to all PCR products. These products were then verified on an agarose gel (the Qiagen) and purified using the S.N.A.P.™ DNA clean-up system (the Qiagen).

An aliquot (2 µl) of purified PCR gel band product was pipetted into a fresh 0.5 ml microcentrifuge tube at room temperature followed by the addition of 0.5 µl pCR®-XL-TOPO® (5 ng plasmid DNA; 50% v/v glycerol; 50 mM Tris-HCl, pH 7.4, 1 mM EDTA; 2 mM DTT; 0.1% v/v Triton X-100; 100 µg/ml BSA; phenol red). The reaction was incubated at room temperature for 5 min, following which the reaction was stopped by adding 0.5 µl of 6x TOPO® cloning stop solution (0.3 M NaCl; 0.06 M $MgCl_2$) and placing the tube on ice. An aliquot of the cloning reaction was used to transform One Shot® TOP ten chemically competent *E. coli* cells (see Section 2.5.3) or One Shot® TOP ten electro-competent (see Section 2.5.4).

Site-directed mutagenesis of a TOPO® clone of the *CAK1* gene used the mutagenic primers in (Table 2.2) and the Stratagene® mutagenesis kit in accordance with its instructions. Sequences were checked by dye-terminator sequencing.

2.5 Handling bacteria

2.5.1 *E. coli* growth media and culture conditions

E. coli cultures were routinely grown in LB broth at 37°C (Bacto-tryptone 1%; yeast extract 0.5%; sodium chloride 1%) with plates of each media made by addition of Bacto-agar 2% prior to autoclaving. *E. coli* transformed with plasmids containing a gene for antibiotic resistance were grown with LB containing the appropriate antibiotic at the relevant working

concentration (ampicillin to a final concentration of 100 µg/ml, kanamycin to a final concentration of 50 µg/ml).

2.5.2 Preparation of competent *E. coli*

Competent *E. coli* cells were prepared according to the calcium chloride technique described in Maniatis *et al*. (1989). The competent cells were resuspended in ice cold 0.1 M $CaCl_2$ plus 15% v/v glycerol and stored in 0.1 ml aliquots at -80°C.

2.5.3 Transformation of chemically competent bacteria

Aliquots of competent *E. coli* cells were thawed on ice and 50 µl pipetted into a chilled 1.5 ml microcentrifuge tube. An appropriate amount (1-100ng) of plasmid DNA or 5-10 µl of vector ligation reaction was added to the chilled cells, mixed gently by pipetting and incubated on ice for 30 min. The cells were then heat-shocked at 42°C for 90 s and placed on ice a further 3 min to recover. The cells were then resuspended in 500 µl of SOC medium (2% w/v tryptone; 0.5% w/v yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 20 mM $MgSO_4$; 20 mM D-glucose) and incubated at 37°C for 1 h. Cells were pelleted at 3,000 xg for 30 s. The pellet was then resuspended in 100 µl of the supernatant and then plated on LB agar containing the appropriate antibiotic before incubation at 37°C overnight.

2.5.4 Preparation of plasmid DNA from *E. coli*

For mini-preps, single bacterial colonies were inoculated into 5 ml LB supplemented with the appropriate antibiotic (for midi-preps 50 ml LB). The cultures were incubated overnight at 37°C with shaking to allow bacterial growth. To purify plasmid DNA the appropriate QIAprep® Miniprep or Midiprep kits were used as described in the appropriate QIAprep® handbooks (Qiagen Ltd.) to obtain approximately 20-200 µg of high-copy plasmid DNA, respectively.

2.6 Biochemical techniques

2.6.1 Extraction of total cell protein

The yeast strain required was grown in 100 ml of the appropriate media from an OD_{600} of around 0.2 to an OD_{600} of around 1.0 at 30°C with agitation followed by the relevant stress conditions for 1 h if required. Cells were pelleted in 50 ml centrifuge tubes by centrifugation (3,000 xg, 5 min) and the supernatant was discarded. Pellets were washed twice with 25 ml dH_2O and transferred to 2 ml screw-cap tubes. Two volumes of acid washed glass beads (Sigma-Aldrich Company Ltd.) were added to the cell pellet. Protein extraction buffer (50 mM Tris-HCl, pH 8.0; 1 complete, EDTA-free tablet (Roche Diagnostics Ltd.) per 50 ml extraction buffer) was added at a volume sufficient to cover the cell/bead suspension. Cells were disrupted by bead-beating (MiniBeatbeater-8™, BioSpec Products Inc., Oklahoma, US) for 30s before chilling on ice for 30s. This procedure was repeated 4 times to ensure complete cell breakage. Beads and cellular debris were removed by centrifugation (13,000 xg, 8 min, 4°C) and soluble protein-rich supernatant was removed and transferred to a fresh 1.5 ml microcentrifuge tube.

2.6.2 Determination of protein concentration

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) which is based on the Bradford assay. This colourimetric protein assay works due to the blue dye primarily binding basic and aromatic amino acid residues, especially arginine, and the subsequent spectrophotometer 595 nm measurements being compared to a BSA standard curve. Both standard and unknown protein concentrations were assayed as by manufacturer's instructions.

2.6.3 Purification of 6x histidine-tagged proteins using Talon® beads

Talon® beads (Takara Bio Europe/Clontech Inc.), which are precharged with cobalt ions (Co^{2+}), were also used to purify the 6x His-tagged protein of interest from the total soluble protein-rich extract (the beads had been washed 3 times with 1 ml of protein extraction buffer and resuspended in the original volume using the same buffer). A suitable volume of soluble protein extract was incubated with 50 µl Talon® beads in a 1.5 ml microcentrifuge tube. Tubes were then incubated at room temperature for 5 min, inverting them every few minutes to mix. After incubation the tubes were spun down at 3,000 xg for 30 s and the supernatant was removed. The beads were then washed five times with wash buffer (20 mM sodium phosphate; 500 mM NaCl; 20-40 mM Imidazole, pH 7.4). The supernatant was removed after the last wash and the beads were resuspended in protein extraction buffer. Protein loading buffer (0.063 M Tris-HCl, pH 6.8; 10% v/v glycerol; 5% v/v β-mercaptoethanol; 0.2% w/v SDS; 0.025% w/v bromophenol blue) was added to the samples and tubes were boiled at 95°C for 10 min. This allows the elution of the 6x His-tagged protein from the beads since the heat breaks the protein-bead bond. After incubation, the tubes were spun down at 3,000 xg for 30 s and the supernatant was removed and transferred to a fresh 1.5 ml microcentrifuge tube.

2.6.4 Purification of 6xHistidine-tagged proteins using fast performance liquid chromatography (FPLC)

To obtain soluble protein-rich extract, 1-2 L of yeast cells containing the 6xHistidine-tagged protein of interest were grown to OD_{600}= 1.0 in suitable induction selective media at 30°C shaking at 160 rpm and harvested by centrifugation at 5000 xg, 5 min. The pellet was resuspended in an equal volume of binding buffer, A (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). The yeast cells were broken by passing through a Cell

Disrurtion System (Constant Cell Disruption Systems, Northants, UK) at 30psi pressure. The extract was centrifuged at 8000 xg for 5 min. The protein rich supernatant was then removed and the lysate was cleared of debris by filter steralising it. Protein concentration was determined by BioRad assay. The soluble protein extract was applied to HisTrapTM FF crude 5 ml column containing Ni^{2+} SepharoseTM 6 Fast Flow (GE Healthcare Life Sciences 17-5255-01). The column was washed with 5 bed volumes of binding buffer, A. An imidazole step gradient was applied to the column to elute the proteins of interest by increasing the percentage of Elution buffer, B, (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) to binding buffer A. Fraction samples were collected and resolved using SDS/PAGE for analysis by western blot and staining with Coomassie blue R-250.

2.6.5 Separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After assembly of 1.5 mm glass plates in the mounting apparatus, the resolving gel was cast using the appropriate volume of 30% acrylamide:bis-acrylamide (29.2% w/v acrylamide; 0.8% w/v bisacrylamide) (Geneflow Ltd., Staffordshire, UK) to make up the resolving gel stock solution (500 mM Tris-HCl, pH 8.8; 0.1% w/v SDS) (Geneflow Ltd.) and polymerised upon addition of 0.1% v/v TEMED (Sigma-Aldrich Company Ltd.) and 1% v/v APS (10% w/v ammonium persulphate). Once the resolving gel had set it was overlaid with 4% acrylamide:bis-acrylamide stacking gel stock solution (125 mM Tris-HCl, pH 6.8; 0.1% w/v SDS) (Geneflow Ltd.), which had polymerisation initiated by addition of 0.1% v/v TEMED and 1% v/v APS, and the relevant gel combs were inserted. After the stacking gel had set the completed gel was fitted into the Bio-Rad Mini-Protean-3® electrophoresis tank as described in the manufacturer's instructions and the tank was filled with 1x SDS-PAGE

running buffer (25 mM Tris; 250 mM glycine; 0.1% w/v SDS). The comb was then removed and the wells created were carefully rinsed with running buffer.

Prior to loading, protein samples were boiled at 95°C for 10 min in an appropriate volume of protein loading buffer (0.063 M Tris-HCl, pH 6.8; 10% v/v glycerol; 5% v/v β-mercaptoethanol; 0.2% w/v SDS; 0.025% w/v bromophenol blue). These samples were loaded into wells formed within the stacking gel, and electrophoresis proceeded at a constant current of 60 mA until the appropriate level of protein separation had occurred. SeeBlue® Plus2 pre-stained standard (Invitrogen Ltd.) was run beside the samples to indicate protein size.

2.7 Analysis of proteins after SDS-PAGE

After electrophoresis was completed the gel was dismantled and the proteins were visualised by one of the following methods.

2.7.1 Direct staining

Staining with Coomassie Brilliant Blue stain solution (0.1% w/v Coomassie Brilliant Blue R-250 (Sigma-Aldrich Company Ltd.); 40% v/v methanol; 10% v/v acetic acid) allows detection of abundant proteins (1µg or more). Gels were incubated for 1 h at room temperature with shaking. The gels were subsequently incubated overnight in destain solution (15% v/v methanol; 7.5% v/v acetic acid) with shaking and several changes of destain until the pale blue background of the gel was removed.

2.7.2 Western blotting

Western blotting is a technique that allows the transfer of SDS-PAGE separated proteins (see Section 2.6.5) from the gel to a nitrocellulose membrane for further analysis. To set up the blot a “sandwich” structure was made consisting of 1 fibre pad (Bio-Rad Laboratories

Ltd.), 2 pieces of 3 mm Whatman® filter paper (Camlab Ltd., Cambridgeshire, UK) cut to the size of the fibre pad, the gel to be blotted, a piece of nitrocellulose membrane (Protran® BA 85, Camlab Ltd.) to cover the gel (air bubbles being removed by smoothing out), and a further 2 pieces of 3 mm filter paper followed by 1 fibre pad. All these components were saturated in chilled transfer buffer (25 mM Tris; 150 mM glycine, pH 8.3; 40% v/v methanol) prior to assembly. The completed "sandwich" was immersed within a transfer buffer-filled Bio-Rad Mini Trans-blot® electrophoretic transfer system (Bio-Rad Laboratories Ltd.), with the gel facing towards the cathode and membrane towards the anode. The tank contained an ice-pack to keep the apparatus cool. A constant current of 200 mA was applied for 2 h in order to transfer the proteins from gel to membrane. Efficiency of protein transfer was verified by staining the membrane with Ponceau-S (Sigma-Aldrich Company Ltd.). The transient stain was water-soluble and did not affect further analysis of blotted proteins. By running pre-stained protein marker during SDS-PAGE, both the efficiency of transfer and molecular weight of sample proteins could be identified.

2.7.3 Identification of immuno-reactive proteins by ECL Plus™ detection

All immunodetection steps were carried out at room temperature with constant agitation. 1x TBS-T (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% v/v Tween-20 Sigma-Aldrich Company Ltd.) was used as a base for blocking solution (1% w/v BSA Sigma-Aldrich Company Ltd. dissolved in TBS-T) in which the membrane produced by Western blotting (see Section 2.7.2) was incubated for 1 h. The membrane was then incubated for 1-4 h, depending on the specificity of the antibody, with agitation in blocking solution containing the required dilution of primary antibody. Three 5 min washes with 1x TBS-T were carried out to remove any unbound primary antibody. The membrane was then incubated for 1 h with agitation in blocking solution containing the required dilution of secondary antibody.

This antibody is conjugated to horse radish peroxidase (HRP). Three 5 min washes with 1x TBS-T were again carried out to remove any unbound secondary antibody. Once the washes were completed the membrane was ready for enhanced chemiluminescence (ECL Plus™) (GE Healthcare Ltd., Buckinghamshire, UK) detection. Western blots were analysed with anti-His, anti-myc, anti-actin, anti GAPDH and anti-PSTAIRE antisera as previously described (Mandal et al., 2007; Millson et al., 2005; Mollapour and Piper, 2007).

This detection method works on the principle that the HRP molecule conjugated to the secondary antibody can react with a luminol substrate found in the ECL Plus™ reagents leading to light being emitted. This light can then be detected by exposure to film. To carry out this detection method the ECL Plus™ reagents were first prepared by mixing, as per manufacturer's instructions, and then applied to the membrane for 2 min. The membrane was then transferred to a film cassette and exposed to Kodak® X-Omat LS film (Sigma-Aldrich Company Ltd.) for between 5 s and 15 min, depending on the intensity of the signal. The film was then developed using an automatic film developer (Compact X4, Xograph Healthcare Ltd., Gloucestershire, UK) and the pre-stained standards were marked.

Chapter 3: Characterisation of the stable complex forming between Cdc37 and the most atypical member of the yeast kinome, Cak1

3.1 Introduction: Background to the studies in this Chapter

3.1.1 Cdc37 with a C-terminal BD extension is functional in yeast

Amongst the Cdc37 proteins of distantly-related species it is only the 20-30 amino acids at the extreme N-terminus of Cdc37 that are highly conserved in sequence (MacLean and Picard, 2003), a strong indication that this N-terminal region is important for Cdc37 function. Consistent with this our collaborators in the Netherlands noted that sequences placed at the N-terminus of Cdc37 generally lead to a loss of Cdc37 functionality. Thus, while Cdc37 overexpression will normally rescue the osmosensitive growth of the *hsp90-G81S* mutant (Yang et al., 2006), overexpressions of N-terminally GFP- or GST-tagged forms of Cdc37 could not provide any rescue of this mutant in the presence of 2M sorbitol even though these proteins have been shown to be stably expressed by western analysis (P. Hawle, unpublished data) (Figure 3.1a). Contrasting with this are the experiences of placing sequences at the C-terminus of Cdc37. Cdc37 has been shown to be functional when tagged at its C-terminus with either GFP, the –HA epitope (Hawle et al., 2007) or the tandem affinity tag (Puig et al., 2001).

A number of potential interactors of Cdc37 have already been identified in experiments involving immunoprecipitation (Abbas-Terki et al., 2000; Hawle et al., 2007), yeast two-hybrid (Y2H) (Lamphere et al., 1997; Millson et al., 2004; Mort-Bontemps-Soret et al., 2002) or the mass spectrometry of protein complexes (Gavin et al., 2002; Krogan et al., 2006). We noted that all the earlier Y2H screens had used “bait” or “prey” fusions where the BD or AD domain of Gal4 is positioned at the N-terminus of Cdc37. Concerned that these

might not have been functional fusions P. Hawle and S. Millson in this laboratory constructed (by homologous recombination in PJ694-α yeast; see Methods), vectors for the expression of Y2H "bait" fusions where the BD domain placed at the C-terminus of either a wild-type or a Cdc37(S14A) mutant Cdc37 (Cdc37-BD; Cdc37(S14A)-BD respectively). After extraction from the original PJ694-α transformants (PH600/1; Table 2.1), the *TRP1* vectors carrying these fusion genes were retransformed into a strain (XX201; Table 2.1) in which the essential Cdc37 function is provided by a *CDC37-GFP* gene carried on a centromeric *URA3* plasmid. Transformants of this XX201 growing on minus tryptophan plates were subsequently streaked onto agar containing 5-fluoroorotic acid (FOA), whereupon only cells able to lose the original *URA3*-based *CDC37* plasmid should grow. As shown in (Figure 3.1b), the cells containing the Cdc37-BD and the Cdc37(S14A)-BD fusion vectors were capable of growth in presence of FOA, indicating that Cdc37 and Cdc37(S14A) are still functional with a C-terminal -BD extension. As is normal for the *cdc37(S14A)* yeast mutant, the cells expressing Cdc37(S14A)-BD as their sole Cdc37 (PH603; Table 2.1) were temperature sensitive (not shown).

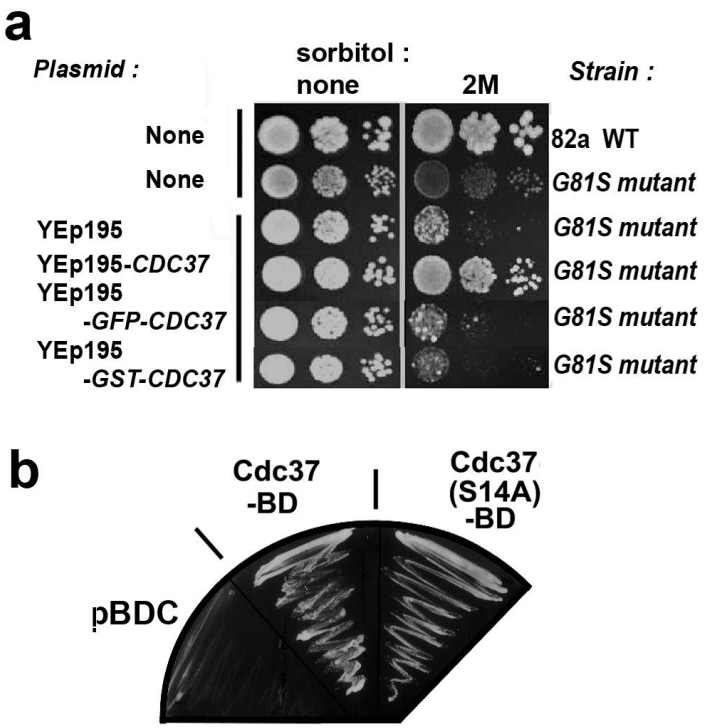


Figure 3.1. **(a) Overexpression of an N-terminally GFP- or GST-tagged Cdc37 does not rescue the osmosensitivity of the *hsp90-G81S* mutant. Serial dilutions of untransformed wild type (82a WT), or osmosensitive *hsp90-G81S* mutant cells; also *hsp90-G81S* cells transformed with the empty multicopy vector YEplac195 or YEplac195-based plasmids with genes either native Cdc37, N-terminally GFP- or GST-tagged Cdc37 were pinned on YPD; also YPD containing 2M sorbitol. Plates were photographed after 3d at 24°C. (b) Growth (3d 30°C) of Trp+ transformants of strain XX201 on FOA, cells containing either empty pBDC vector, or pBDC containing a Cdc37-BD or Cdc37(S14A)-BD gene insert. No growth is apparent with the empty vector pBDC (data generated by Dr P. Hawle).**

3.1.2 A genomic Y2H screen for interactors of Cdc37-BD and Cdc37(S14A)-BD.

In the Y2H study conducted by P. Hawle and S. Millson in this laboratory PJ694α cells expressing either a Cdc37-BD or a Cdc37(S14A)-BD bait fusion (strains PH600/1; Table 2.1) were robotically mated with a previously-described array of AD-fusions to the 6000 different proteins of yeast, the latter expressed in cells of the opposite mating type (PJ694a) (Uetz et al., 2000). Diploids - now expressing either Cdc37(Wt)-BD or Cdc37(S14A)-BD and an AD-fusion protein - were selected on medium lacking tryptophan and leucine, then transferred to medium without tryptophan, leucine and histidine but containing 4mM 3-AT. Activity of the interaction-responsive *HIS3* reporter gene was monitored as 3-AT resistant growth over 4, 8 and 16 days incubation at 30° C. (Figure 3.2a) shows the strong positives appearing on four sample plates, arrayed diploid cells (now expressing both the -AD and the Cdc37(S14A)-BD fusion) grown 16d in the presence of 4mM 3-AT. Eliminating the known false-positives on the array, only a relatively small number of interactors were detected (15 interactions with the Cdc37-BD fusion and 9 with Cdc37(S14A)-BD; P Hawle thesis, 2008).

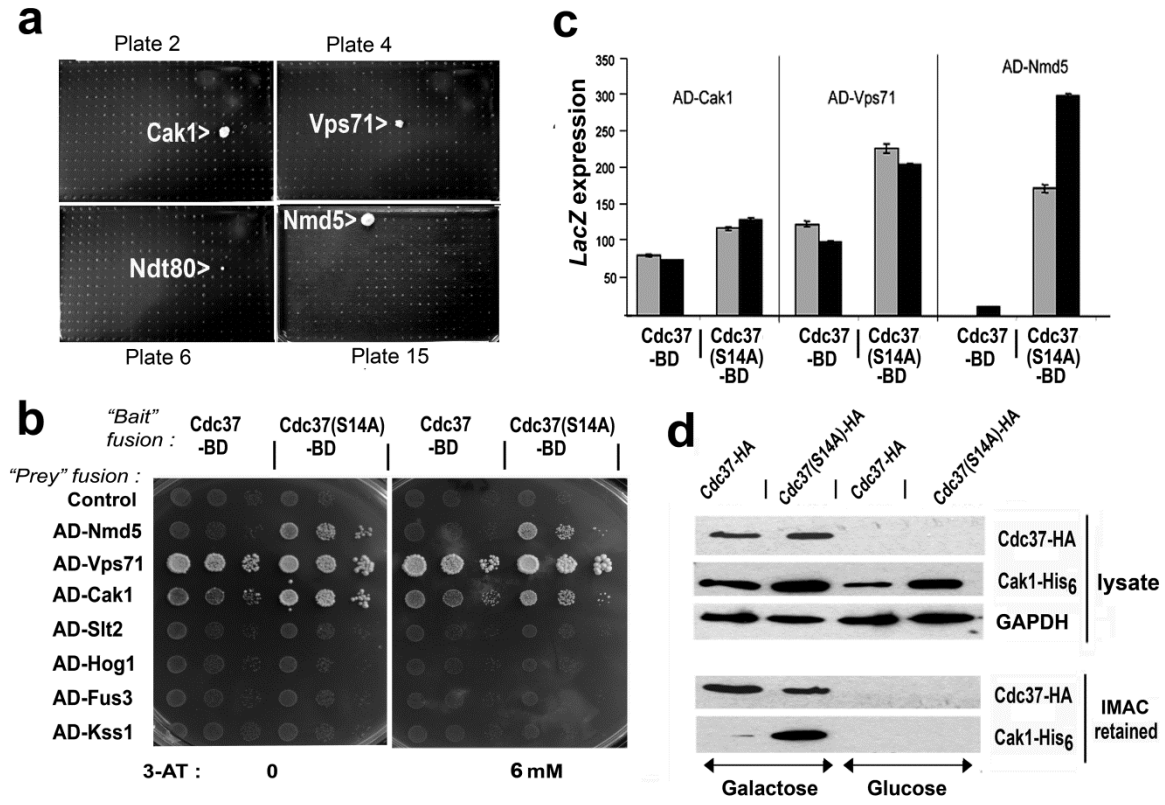


Figure 3.2. (**a) Four sample plates of the 384-colony format 16-plate library array of PJ694a/α cells containing the Cdc37(S14A)-BD bait. Activation of the interaction responsive *HIS3* gene is moitored as 3-AT resistant growth (16 d at 30°C) on SD medium without tryptophan, leucine and histidine containing 4 mM 3-AT; Cdc37(S14A)-BD interactions represented on these plates being: plate 2: AD-Cak1; plate 6: AD-Ndt80; plate 7: AD-Vps71; plate 15: AD-Nmd5. The colony size is an indicator of interaction strength. (b) Serial dilutions of PJ694a/α cells expressing Cdc37-BD or Cdc37(S14A)-BD baits and the indicated AD-fusions after growth (10d at 30°C) on this same medium and either zero or 6mM 3-AT. (c) Measurements of interaction-responsive *LacZ* reporter gene expression in PJ694a/α cells analysed after growth to mid-log phase at 24° (grey), or 1h following a heat shock from 24° to 39°C (black). *LacZ* expression is expressed as percentage increase relative to that of PJ694a/α control cells containing empty pBDC plasmid and the corresponding AD-protein fusion. (d) Total versus His Tag -retained His_6-Cak1, Cdc37-HA and Cdc37(S14A)-HA in extracts from 28°C glucose and galactose-grown cultures of DH211 and DH212 containing pYES2-His_6-CAK1, a vector for *GAL1* promoter-driven overexpression of His_6-Cak1. Blots were probed with anti-HA, anti-His and (as loading control) anti-GAPDH antisera (data in (a) generated by Dr P. Hawle).**

3.2. Results

3.2.1 Confirmation of the Cdc37-BD:AD-Cak1 Y2H interaction

We initiated this thesis study by confirming certain of the interactions identified in Dr Hawle's genomic screening of the Cdc37-BD or the Cdc37(S14A)-BD bait fusions against the library of AD-yeast protein fusions (Figure 3.2a). Repinning (Figure 3.2b) and expression measurements of the interaction-responsive *LacZ* reporter gene in PJ694a/α (Figure 3.2c) confirmed the interactions with AD-Cak1, AD-Vps71 and AD-Nmd5; also that the S14A mutation in the bait was moderately reinforcing the interactions with AD-Cak1 and AD-Vps71 and strongly reinforcing the interaction with AD-Nmd5.

To confirm that Cdc37 associates with Cak1 in cell extracts an N-terminally His_6-tagged Cak1 (His_6-Cak1) was overexpressed from the *GAL1* promoter of plasmid pYES2-His_6-CAK1 in DH211 and DH212 (Table 2.1), strains which possess C-terminally HA-epitope tagged forms of either the wild type or the *cdc37(S14A)* mutant Cdc37 respectively (Cdc37-HA; Cdc37(S14A)-HA). Immobilized metal affinity chromatography (IMAC) retention of the His_6-Cak1 in cell extracts confirmed that Cdc37-HA was being retained by this His_6-Cak1 and that such retention is enhanced with S14A mutation of this Cdc37-HA, a result consistent with the Y2H data (Figure 3.2b-d). We subsequently found this Cak1:Cdc37(S14A) complex to be sufficiently stable as to be isolatable by gel filtration (see below).

3.2.2. Analysis of Cak1 and Cdc37 protein levels in Cdc37 phosphorylation mutants.

Our Y2H screen did not detect most of the protein kinases thought to be Cdc37 "clients" in yeast, for the probable reasons outlined in the Discussion (section 3.3). Remarkably though did detect Cdc37(S14A)-BD interacting with at least two protein kinases (Ste20, Cak1) which would appear, on the basis of current evidence, may not require Cdc37

phosphorylation for their stability (Table 2). An earlier study indicated that the stability of Ste20 is unaffected by *cdc37(S14A),* a mutation that destabilizes much of the yeast kinome (Mandal et al., 2007). Furthermore we have found that the *cdc37(S14A)* mutant can efficiently overexpress His_6-Cak1 (Figure 3.2d). Though Cak1 may be a Cdc37 "client" in yeast (reduced stability of Cak1 having been reported in the temperature sensitive *cdc37-1* mutant (Farrell and Morgan, 2000)), the eukaryotic Cdc37 chaperone function may not be critical for the proper folding of Cak1 since a GST-Cak1 fusion expressed in *E. coli* is fully active and is able to efficiently phosphorylate Cdk (Song et al., 2001).

Cak1 has not been identified as one of the several protein kinases destabilized in *cdc37(S14A)* yeast cells. So that Cak1 levels could be analysed in this, as well as other Cdc37 phosphorylation mutants, the chromosomal *CAK1* coding sequence of strains DH211-5 was *myc* epitope tagged using the *13myc-TRP1* cassette system (Table 2.1 and Section 2.2). Analysing protein extracts from these cells (Figure 3.3), the levels of Cak1-*myc* in the *cdc37(S14A)* mutant did not appear to be as dramatically reduced as those of Cdc28 (the latter Cdk, a major phosphorylation target of Cak1, is destabilized by the *cdc37(S14A)* mutation (Mandal et al., 2007)). Levels of Cak1-*myc* were also substantially unaltered in cells expressing the nonphosphorylatable S17A and phosphomimic (S14E, S17E) mutant versions of Cdc37-HA (Figure 3.3). This reveals that, whereas Cdc37 is probably required for the *in vivo* stability of Cak1 (since Cak1 levels are reduced in the *cdc37-1* mutant (Farrell and Morgan, 2000)), this stabilization is not dependent on the CK2 phosphorylation/Ppt1 dephosphorylation of Cdc37 (Vaughan et al., 2008).

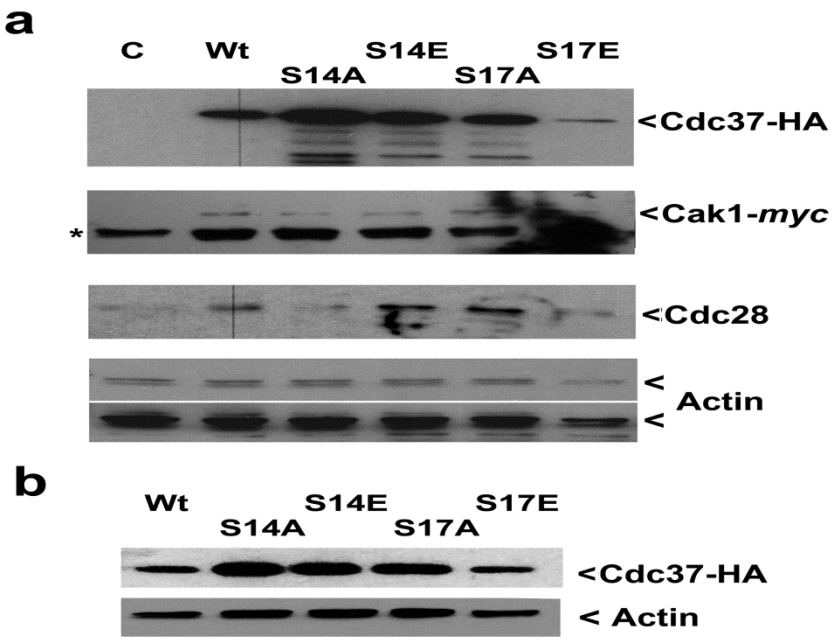


Figure 3.3 (a) Analysis of Cak1 and Cdc28 in Cdc37 phosphorylation mutants. Protein extracts from 24°C cultures of strains DH211-5 CAK1-*myc* were western blotted and probed using anti-HA, anti-myc, Pstaire and anti-actin (loading control) antisera. Two exposures of the actin blot are shown; a nonspecific band from the anti-myc probing is indicated with an asterisk (*). The control lane (C) is an identically-treated extract from strain TM141 in which neither the Cdc37 nor the Cak1 is epitope tagged. (b) An analysis of Cdc37-HA levels in the original DH211-5 (Hawle et al., 2007).

3.2.3 Cells expressing S14A, S14E, S17A mutant versions of Cdc37 have elevated levels of Cdc37.

While conducting this analysis we were intrigued to find that levels of Cdc37-HA are elevated in yeast strains expressing, as their sole Cdc37, the S14A, S14E, S17A mutant versions of this Cdc37-HA. As shown in (Figure 3.3), levels of Cdc37-HA are increased in strains DH212-4 CAK1-myc, relative to the cells expressing either the wild type or S17E mutant Cdc37-HA (strains DH211 CAK1-myc and DH215 CAK1-myc). The reasons why these mutations in Cdc37 increase cellular levels of this chaperone have yet to be established. However it should be noted that these Cdc37 mutations, in contrast to S17E, also render cells highly sensitive to stress and the loss of Hsp90 activity (Bandhakavi et al., 2003; Vaughan et al., 2008).

3.2.4 Purification of Cak1 in complex with Cdc37(S14A).

Cak1 has previously been found to associate with both Cdc37 and Cdc28 in yeast (Lamphere et al., 1997; Millson et al., 2004; Mort-Bontemps-Soret et al., 2002). In this study we identified that Cak1:Cdc37 association was enhanced by the presence of the S14A mutation in Cdc37 (Figure 3.2b-d), despite this mutation normally acting to destabilise protein kinases. To further characterize the protein composition of what appeared to be a relatively stable complex forming *in vivo* between Cak1 and Cdc37(S14A) we modified an *S. cerevisiae* vector autoselection system for recombinant protein expression (Geymonat et al., 2007), so that the cells would simultaneously overexpress both of these yeast proteins (also the native Cdc28 and Mob1) during growth on galactose.

The published vectors for this expression system all create proteins with N-terminal sequence extensions (His_6- or glutathione transferase (GST-)). However since such N-terminal sequence extensions generally inactivate Cdc37 (Figure 3.1), Dr Millson in this

laboratory modified the *TRP1/MOB1* vector pMH903 (Geymonat et al., 2007) so that it had a unique Nde1 site upstream of the GST gene (plasmid pMH903(Nde1)) and so that homologous recombination in yeast using this Nde1-cleaved vector could generate a *GAL1* promoter regulated gene for expressing a Cdc37-precission-Streptag fusion protein in which the N-terminus of Cdc37 was preserved intact.

- ***Strains with essential genes (MOB1, CDC28) on maintenance plasmids :-***

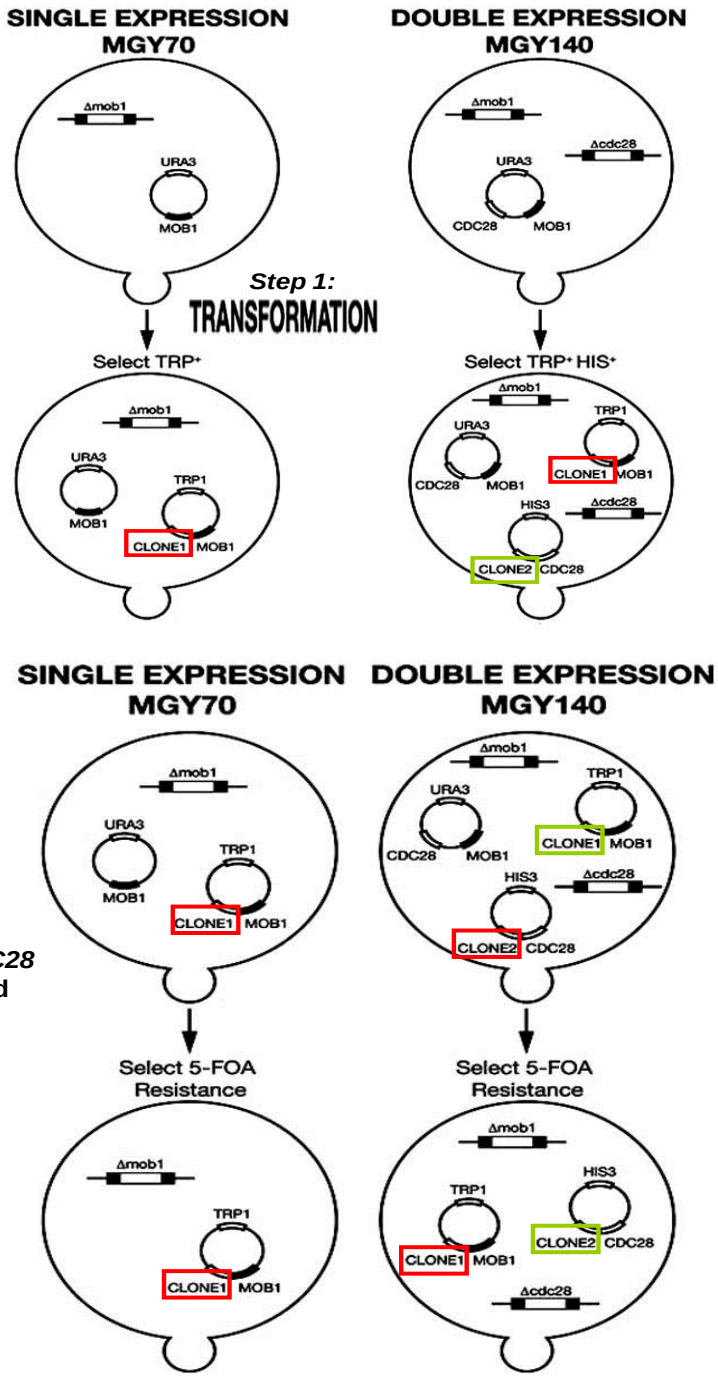


Figure 3.4 The *S. cerevisiae* plasmid autoselection system for maintaining vectors for optimised expression of a single recombinant protein (strain MGY70) or two recombinant proteins (strain MGY140) (adapted from (Geymonat et al., 2007)).

Strain MGY140 (Table 2.1) was engineered (see Figure 3.4 and Methods) so that it maintained two expression vectors, each with a bidirectional *GAL1* promoter regulating two genes: (1) a pMH940 derivative bearing genes for Cdc28 and His_6-TEV-Cak1 (Cak1 with a N-terminal TEV protease-cleavable His_6 tag), also (2) a pMH903(Nde1) derivative bearing genes for Mob1 and Cdc37(S14A)-Strep (the latter Cdc37(S14A) with a C-terminal, precision protease-cleavable Strep2 tag).

Following growth on galactose and cell breakage, we isolated the His_6-Cak1 in the cell extract on His Tag resin. This His_6-Cak was found to be associated with a substantial amount of Cdc37(S14A)-Strep, but very little Cdc28 and no detectable Hsp90 (Figure 3.5). Gel filtration of the His Tag -eluted sample applied to a 16/60HiLoadSuperdex 200 column equilibrated with PBS buffer indicated it contained a substantial amount of free His_6-Cak1 (yield ~20mg/litre culture), as well as – at lower level – complexes containing His_6-Cak1, Cdc37(S14A) and a little Cdc28 (Figure 3.5). Much of it was a complex with an apparent MW of 165kDa, indicative of a monomer of Cak1 in association with a dimer of Cdc37(S14A).

When this His Tag -eluted protein was subsequently applied to streptavidin agarose resin, there was no selective retention of the His_6-Cak1 (though this resin bound the free Cdc37(S14A)-Strep in cell extracts; not shown). This is an indication that the C-terminal Strep2 tag on the Cdc37(S14A) bound to His_6-Cak1 may be buried in the structure of the His_6-Cak1:Cdc37(S14A)-Strep complex and that for further affinity purification of this complex it would be expeditious to use a longer or larger affinity tag at the C-terminus of Cdc37. Such tagging of Cdc37 with the tandem affinity tag has already been used with success (Puig et al., 2001).

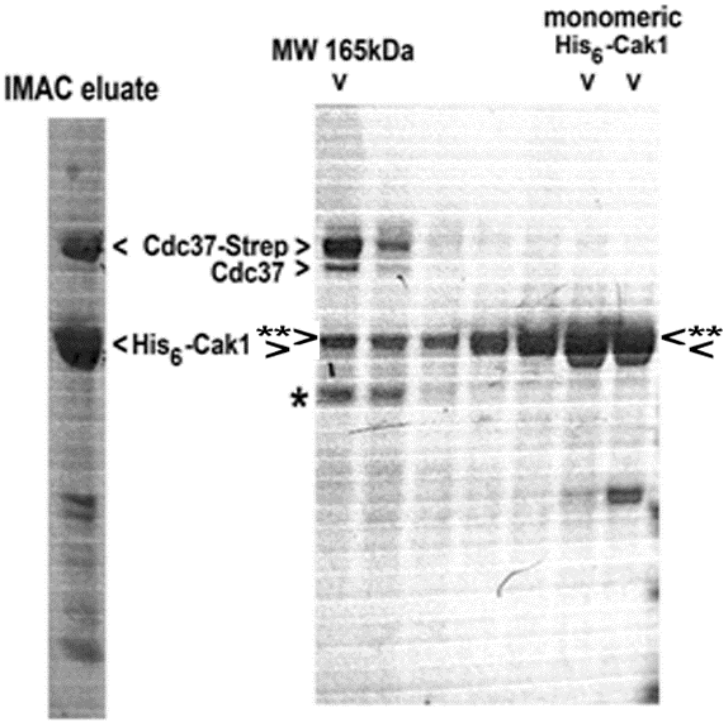


Figure 3.5 Production of 6xHis-TEV-CAK1 at ~20mg/litre (also – at lower level the His_6-TEV-Cak1/Cdc37-Strep complex and a little 6xHis-TEV-CAK1/Cdc28/Cdc37 complex) by overexpression in galactose-grown MGY140 cells. A total elute of the His Tag -retained material (left) analysed by gel filtration (right), revealing both resin-included material (His_6-TEV-CAK1) and a 165kDa peak formed substantially of His_6-TEV-Cak1 and Cdc37-Strep (also a little untagged Cdc37 derived from the chromosomal *CDC37* gene of strain MGY140 and Cdc28(*)). The slower gel migration for the His_6-TEV-Cak1 in Cdc37-Strep association is indicated (**). Shown is a Coomassie stained gel. The identity of the major bands was confirmed by Dr S. Millson in this laboratory, western blotting these fractions and probing with anti His, and PSTAIRE and anti Cdc37 antibodies (not shown).

3.3 Discussion

The novelty of this study is that it has provided the first example of a Cdc37 client kinase whose stable association with Cdc37 and expression level is independent of the CK2 phosphorylation of Cdc37. Stability of Cak1 is greatly reduced in the temperature sensitive *cdc37-1* mutant (Farrell and Morgan, 2000) yet, despite phosphorylation on the conserved S14 of Cdc37 being crucial for the stability of most protein kinases in yeast (Mandal et al., 2007), the interaction of Cak1 with Cdc37 and the expression level of Cak1 *in vivo* appear independent of this phosphorylation (Figures 3.2,3.3). This is a protein kinase that appears not to require the CK2 phosphorylation/Ppt1 dephosphorylation of Cdc37 that is so vital for stabilization of much of the kinome. Not only were we able to overexpress His_6-Cak1 efficiently in the *cdc37(S14A)* mutant (Figure 3.2d), but we found that the Cak1 expression level in this mutant was practically normal (Figure 3.3). Though not investigated here, novel structural features of fungal Cak1 (see below) may be a factor in these unusual properties of the Cdc37:Cak1 complex.

Earlier Y2H screens for Cdc37 interactors (Lamphere et al., 1997; Millson et al., 2004; Mort-Bontemps-Soret et al., 2002) all used bait or prey fusions in which the Gal4-BD or AD sequence is positioned at this N-terminus of Cdc37. However it appears that fusion proteins where domains are placed at the N-terminus of Cdc37 are unable to provide the essential Cdc37 function in yeast (Figure 3.1a). Instead fusions in which the –BD is placed at the C-terminus of Cdc37 remain functional (Figure 3.1b) and should therefore potentially be more suitable for Y2H screening. Using such functional Cdc37-BD and Cdc37(S14A) baits in genomic Y2H screens, P. Hawle and S. Millson in this laboratory could identifyonly a few of the 115 protein kinases of yeast as Cdc37 interactors, despite Cdc37 apparently stabilising ~70% of the yeast kinome (based on the evidence of kinase destabilisation in the

cdc37(S14A) yeast mutant (Mandal et al., 2007)). Just two protein kinases exhibited a reasonably strong interaction with Cdc37 (Hal5 and Cak1), with two others displaying weaker interactions (Ste20 and Rim15); P Hawle thesis, 2008). Repinning confirmed that Cdc37-BD and Cdc37(S14A)-BD interact with AD-Cak1, but the lack of any detectable interaction with four yeast MAP kinases (Slt2, Hog1, Fus3, Kss1; Figure 3.2b) previously been shown to interact with Cdc37 in immunoprecipitation studies (Hawle et al., 2007). The reason that these protein kinases known to require Cdc37 for their stability (Hawle et al., 2007; Mandal et al., 2007) were not detected is probably that Cdc37 interactions with nascent protein kinases are generally highly transient in the environment of the living cell (the Y2H system only detects relatively stable *in vivo* protein-protein interaction (Bartel and Fields, 1997)). Indeed it is conceivable that the isolation of reasonably stable complexes between Cdc37 and protein kinases (Hawle et al., 2007; Vaughan et al., 2006) may require the dilution of cell contents that occurs upon cell breakage.

The phenotype of *cdc37(S14A)* cells (Bandhakavi et al., 2003) is remarkably similar to that generated with loss of Cak1 activity (*cak1-ts* mutants become elongated at high temperature, most of the budded cells having a short spindle spanning an undivided nucleus at the mother/bud neck, characteristic of G2 arrest (Kaldis et al., 1996)). Indications of limiting Cdc28 activity being a major factor in the *cdc37(S14A)* phenotype come from its rescue by Cdc28 overexpression or with the loss of Swe1 tyrosine kinase (Bandhakavi et al., 2003). Generally these effects have been attributed to the well-established Cdc37-dependence of Cdc28 itself (Mandal et al., 2007; Turnbull et al., 2005), rather than the loss of Cak1 activity. Consistent with this we were unable to obtain rescue of either the temperature-sensitivity, or the elongated cell phenotype of *cdc37(S14A)* cells with either the His_6-Cak1 overexpression from the *GAL1* promoter of plasmid pYES2-His_6-CAK1 or the expression of a Cak1-independent mutant allele of Cdk that overrides the essential requirement for Cak1 (Cdc28-

42344; (Cross and Levine, 1998))(data not shown). The analysis in this Chapter did though reveal that the S14A, S14E, S17A mutations in Cdc37 operate to increase the cellular levels of this chaperone (Figure 3.3), the first time this effect has been noted. There is currently considerable interest in the factors that might influence the cellular levels of Cdc37, since this is a protein vital for the activity of several of the oncoprotein "drivers" of cancer (Pearl, 2005; Smith and Workman, 2009).

The unusual strength of the Cdc37(S14A) association with Cak1 (Figure 3.2) may reflect the novel structural features of Cak1 protein kinase. In man and in most eukaryotes Cak is a heterotrimeric complex (Cdk7-cyclin H-Mat1) its catalytic subunit (Cdk7) being activated by either phosphorylation of its own activating threonine (Thr-170 in human Cdk7) or the binding to MAT1. In contrast *S. cerevisiae* Cak1 has a highly atypical sequence and is active as a monomer (its activity not requiring cyclin binding, phosphorylation, or an assembly factor *in vivo* (Kaldis et al., 1996; Tsakraklides and Solomon, 2002)). Active throughout the cell cycle, the *S. cerevisiae* Cak1 has unusual biochemical properties and a substrate specificity that are not shared by the human Cdk7. Thus while human Cdk7 prefers cyclin-cdk complexes, these yeast CAKs prefer monomeric cyclin-free Cdk substrates. Human Cdks bind cyclin prior to becoming phosphorylated by Cdk7 whereas in the *S. cerevisiae* cell cycle Cdk is phosphorylated by CAK prior to cyclin binding (Kaldis et al., 1998; Kaldis et al., 1996; Tsakraklides and Solomon, 2002). This difference potentially makes fungal Cak1 a promising target for antifungal drug development.

Structurally fungal Cak1 lacks features conserved in practically all other protein kinases, notably the canonical "glycine loop" motif (G*X*G*XX*G) that normally stabilises ATP in the nucleotide binding pocket as well as a number of highly conserved core residues. Furthermore, fungal Cak is also unique in that - while it contains the "invariant lysine" that

aligns ATP by interacting with its α- and β-phosphates and is essential for catalysis in practically all other protein kinases, this lysine is completely dispensable for their activity both *in vitro* and *in vivo* (fungal Cak is insensitive to the protein kinase inhibitor 5'-fluorosulfonylbenzoyladenosine which, by covalently modifying this lysine, leads to loss of activity in nearly all protein kinases, including Cdk7). Despite this, fungal CAKs still have high affinity for ATP, with a K_m (ATP) ~1.8-5.0μM (Enke et al., 2000; Tsakraklides and Solomon, 2002), indicating that they have compensated for their lack of a glycine loop. It is tempting to speculate that these unusual structural features may contribute to the Cak1:Cdc37 association. Structural analysis of the Cak1:Cdc37 complex in Figure 4 should reveal this, as well as how the Cdc37 N-terminus – the region of Cdc37 highly conserved in evolution – interacts with protein kinases at the atomic level. It also promises to assist the development of drugs that selectively inhibit the fungal, not human, Cak1.

Chapter 4: Analysis of Cak1 phosphorylation

4. 1 Introduction

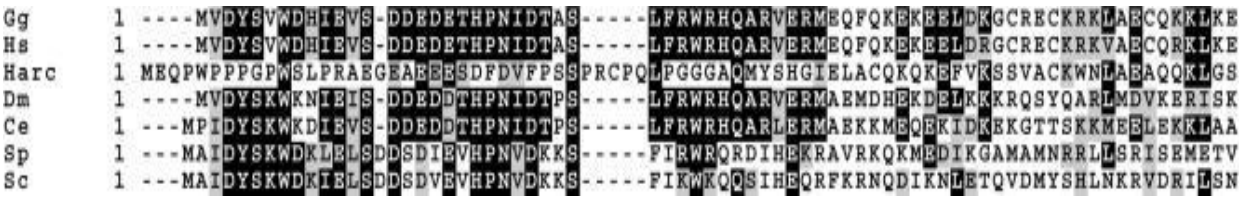
Cak1 is generally thought to be constitutively active throughout the yeast cell cycle and is also reported to be fully active when prepared by *E. coli* expression (Song et al., 2001). As a result there has been no study to date of any possible regulation of this kinase exerted through covalent modification and no systematic study of the role Cak1 phosphorylation events in yeast. Our purification of the His_6-TEV-Cak1/Cdc37-Strep complex overexpressed in galactose-grown MGY140 cells revealed a mobility shift for the His_6-TEV-Cak1 in this complex, as compared to the free His_6-TEV-Cak1 in cell extracts (Figure 3.5). Finding this apparent alteration to the phosphorylation status of the Cak1 in this overexpressed His_6-TEV-Cak1/Cdc37-Strep complex we proceeded to identify the sites of Cak1 phosphorylation in this protein kinase fraction. We then analysed the effects of expressing, as sole essential Cak1 in yeast, forms of Cak1 in which each of these phosphorylated residues was mutated to either a nonphosphorylatable alanine residue or to a phosphomimetic residue.

4.2 Results

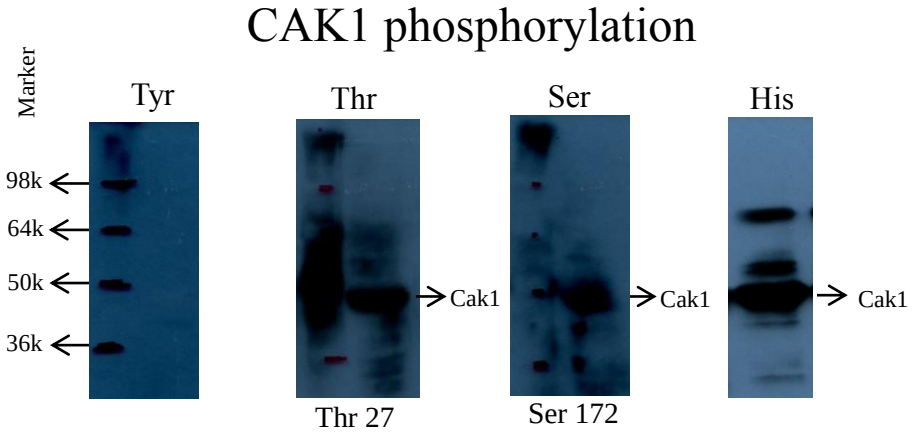
4.2.1 Identification of Cak1 phosphorylations.

When we overexpressed His_6-TEV-Cak1 and Cdc37-Strep in yeast (Section 3.2.4) we noted that the His_6-TEV-Cak1 fraction in association with Cdc37(S14A) was exhibiting a slower gel migration compared to the free His_6-TEV-Cak1 (Figure 3.5). This was due to phosphorylation of this His_6-TEV-Cak1 fraction (not shown). By western blot analysis it was investigated whether this Cak1 fraction in Cdc37(S14A) association cross reacted with anti-phospho Tyr, phosphor-Ser and phospho-Thr antibodies. The results indicated this Cak1 fraction was phosphorylated on both serine and threonine (Figure 4.1).

A sample of the His_6-TEV-Cak1/Cdc37-Strep complex was sent for mass spectrometry analysis to Dr A. Truman, University of Chicago. This detected phosphorylations of the Cdc37 on Ser 173 and Ser 367 (not analysed further in this study) as well as phosphorylations of the His_6-Cak1 at Thr 27 and Ser 172 (the latter within a Ser-Pro motif). Previous analyses of the yeast phosphoproteome had only detected phosphorylation of Cak1 at Ser 205 of the activation loop (Albuquerque et al., 2008).



Clustal W alignment of N-terminal Cdc37 sequences. Sequences corresponding to the 70–80 N-terminal amino acids from 6 different species (Gg, chicken; Hs, human; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sp, *Schizosaccharomyces pombe*; and Sc, *Saccharomyces cerevisiae*), also Cdc37 and the Cdc37 relative Harc from humans. Residues of identity and similarity are shown in black and gray, respectively (from Maclean and Picard, 2003).




```
1    MKLDSIDITH CQLVKSTRTA RIYRSDTYAI KCLALDFDIP PHNAKFEVSI
51   LNKLGNKCKH ILPLLESKAT DNNDLLLLFP FEEMNLYEFM QMHYKRDRRK
101  KNPYYDLLNP SIPIVADPPV QKYTNQLDVN RYSLSFFRQM VEGIAFLHEN
151  KIIHRDIKPQ NIMLTNNTST VSPKLYIIDF GISYDMANNS QTSAEPMDSK
201  VTDISTGIYK APEVLFGVKC YDGGVDVWSL LIIISQWFQR ETSRMGHVPA
251  MIDDGSDDMN SDGSDFRLIC SIFEKLGIPS IQKWEEVAQH GSVDAFVGMF
301  GADGDGKYVL DQEKDVQISI VERNMPRLDE IADVKVKQKF INCILGMVSF
351  SPNERWSCQR ILQELEKP
```

Figure 4.1 Analysis of the aminoacids phosphorylated the His_6-Cak1 in complex with Cdc37(S14A). Phosphorylation on threonine and serine detected by anti-phosphothreonine and antiphosphoserine antibodies corresponded to the His_6-Cak1 signal detected by the anti-His antibody (upper figure). This sample was subsequently analysed by mass spectrometry by Dr A. Truman and found to be phosphorylated at the residues underlined (lower figure).

4.2.2 Construction of strains expressing a Cak1 mutated in each phosphorylation site.

To determine if these phosphorylations at T27, S172 or S205 are of importance for the essential functions Cak1 in yeast, also for the Cak1:Cdc37 association, these residues were individually mutated in a TOPO clone of *CAK1* to either the nonphosphorylatable residue alanine, or to a phosphomimic residue (D or E) (see Methods). By recombinational cloning of these genes with the linearised *LEU2* vector pHSCprom (Panaretou et al., 1999) in strain SY80 (Table 2.1), selecting for Leu+ transformants, then elimination of the original *CAK1/URA3* vector of these Leu+ SY80 cells by growth on FOA, strains were prepared in which these point mutant forms of Cak1 constitute the only form of Cak1 (Figure 4.2a). While all of the introduced mutations were compatible with the essential function of Cak1, the S205E mutant cells grew slowly on the FOA plate (Figure 4.2a). Further investigation of these seven strains, each cured of their original *CAK1/URA3* vector and now expressing a wild type or point mutant form of Cak1, revealed a number of phenotypes. Mutations S205E and T172A rendered the cells slightly temperature sensitive, while T172A resulted in a pronounced respiration deficiency – the first time such deficiency has been found as a consequence of Cak1 mutation (Figure 4.2b).

These seven strains were also sent to Dr A. Truman, University of Chicago for FACS analysis. His analysis of YPD cultures revealed that some of these mutations are affecting progression through the yeast cell cycle (Figure 4.3). However it should be noted that these cells are probably massively overexpressing Cak1 from the strong pHSC82 promoter of plasmid pHSCprom, an overexpression that may have affected the phenotypes observed. We subsequently found that the normal levels of Cak1 in yeast are relatively low (see Figure 3.3a).

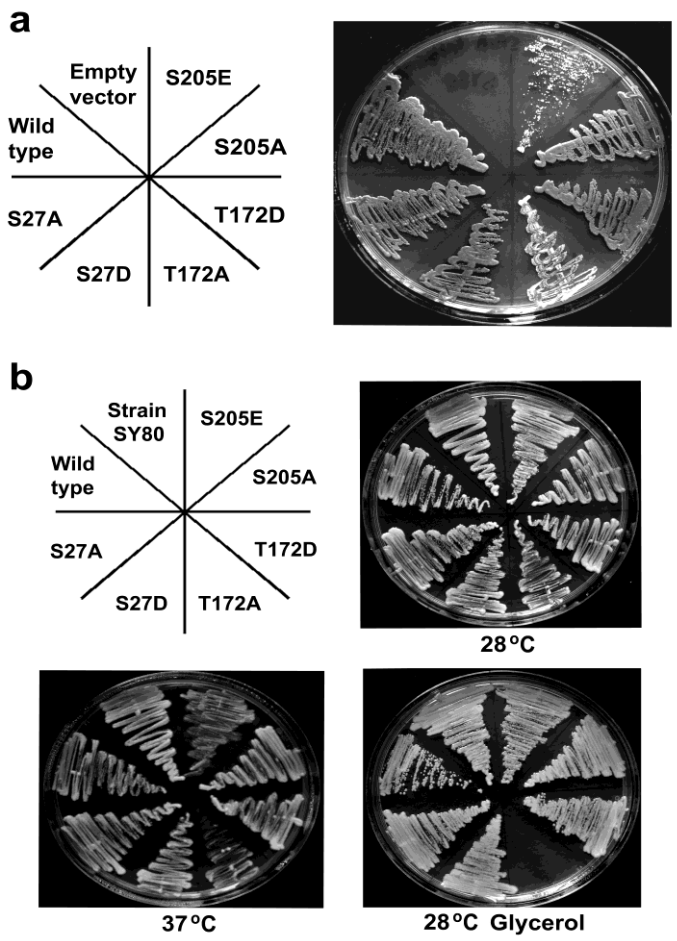
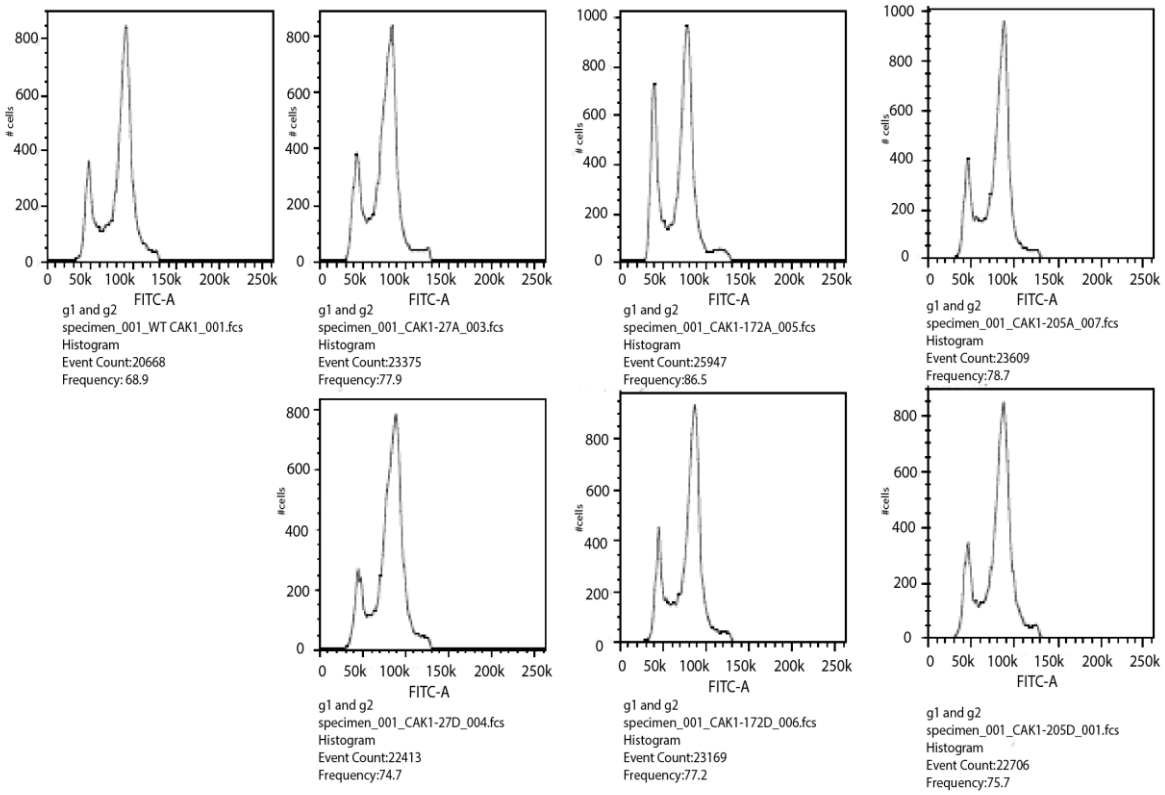


Figure 4.2 (a) FOA growth (4d 28°C) curing of the *CAK1/URA3* vector in SY80 cells transformed either with empty *LEU2* vector, or *LEU2* vector containing a wild type or mutant CAK1 gene. (b) Glucose (2d 28°C, 37°C) or glycerol (5d 28°C) growth of cells expressing wild type or point mutant forms of Cak1.



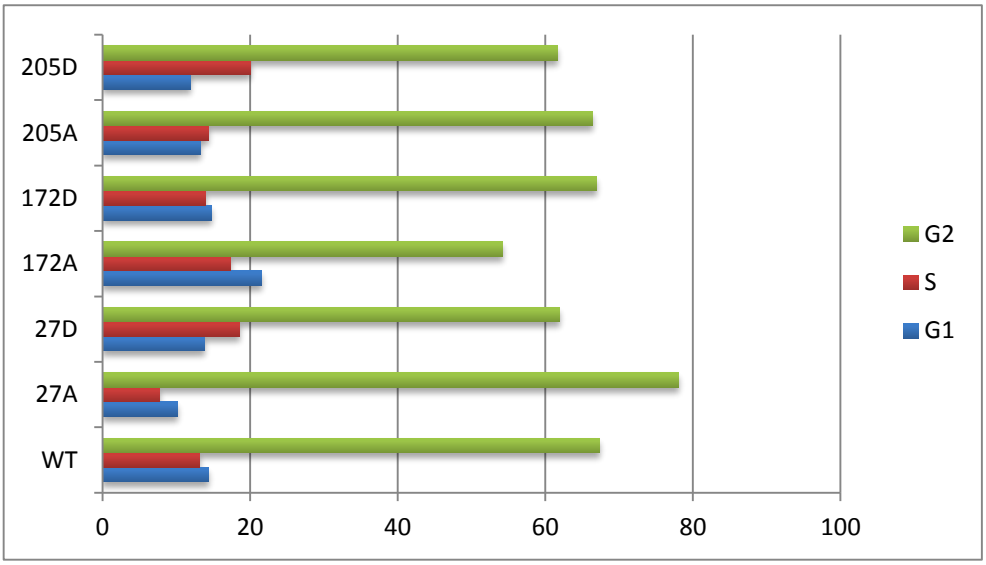


Figure 4.3 Fluoresence activated cell sorting (FACS) analysis of nonsynchronised YPD cultures of SY80 cells expressing different phosphomutant forms of Cak1 (data provided by Dr A. Truman, University of Chicago). This is data from a single experiment, the estimation of relative proportions of cells in the different phases of the cell cycle being shown in the lower diagram.

4.3 Discussion

The analysis in this chapter identified some hitherto unidentified sites of phosphorylation of yeast Cak1. So far these sites have only been shown to be phosphorylated in the Cak1 of the overexpressed His_6-TEV-Cak1/Cdc37-Strep complex of galactose-grown MGY140 cells (Figures 3.5 and 4.1). There is as yet no firm evidence for fungal Cak1 being regulated by phosphorylation, so it remains to be seen whether the phosphorylations of Thr 27, Ser 172 or Ser205 are functionally important, or influence the ability to form the Cak1/Cdc37 complex studied in Chapter 3.

Here, versions of strain SY80 were made in which the sole Cak1 is a form in which each of these residues is either a nonphosphorylatable alanine, or a phosphomimetic residue. While these strains exhibited some marked phenotypes (Figures 4.2 and 4.3), it should be noted that they are almost certainly massively overexpressing the relevant point mutant form of Cak1 from the strong pHSC82 promoter of plasmid pHSCprom. Not only might this have affected the phenotypes observed, but also – through titration effects – it might influence the results of pull-down experiments showing how these mutations influence the protein associations of Cak1. Probably therefore this analysis should be repeated using cells in which these mutant forms of Cak1 are expressed in cells as sole forms of Cak1 from the native *CAK1* gene promoter. Time constraints did not allow this to be done. Neither did it allow us to analyse how these phosphorylation site mutations affect the formation of the Cak1/Cdc37 complex identified in Chapter 3.

Chapter 5: Cdc37 as a target of molybdate and oxidative stress

5. 1 Introduction: Background to the studies in this Chapter

The analysis in Chapter 3 revealed that the presence of the S14A, S14E, S17A mutations in Cdc37 operates to increase cellular levels of this chaperone, the first time that this effect has been noted. Levels of Cdc37-HA were elevated in the strains bearing these mutations (DH212-4), relative to the cells expressing either the wild type or the S17E mutant Cdc37-HA (strains DH211/5) (Figure 3.3). There is currently considerable interest in the factors that might influence the cellular level of Cdc37, since this is a protein vital for the activity of several of the oncoprotein "drivers" of cancer (Pearl, 2005; Smith and Workman, 2009). In certain mammalian cell types an overexpression of this protein is, by itself, oncogenic (Pearl, 2005). Therefore the studies described in this Chapter were initiated in order to investigate whether Cdc37 is an intrinsically stable protein.

Unlike S17E, these Cdc37 mutations (S14A, S14E, S17A) also render cells highly sensitive to stress and the loss of Hsp90 activity (Bandhakavi et al., 2003; Vaughan et al., 2008). Though all three of them sensitise cells to the antibiotics radicicol and geldanamycin (Figure 5.3b), Hsp90 inhibitors that induce an ADP/ATP-free state of the chaperone with low affinity for its client substrates (Prodromou et al., 1997; Roe et al., 1999), S14A would appear to cause the greatest sensitivity to these antibiotics (Figure 5.3b). Earlier work in our laboratory also revealed S14A causes the greatest sensitivity to molybdate (Millson et al., 2009), an inhibitor that is thought to act at a later stage in the chaperone cycle when the client protein is much more tightly bound to the Hsp90 machine and to induce a "locked" form of the client-chaperone complex where the client is stabilised through strong hydrophobic interactions with the ADP/ATP-bound state of the chaperone (Hartson et al., 1999; Pratt and

Toft, 1997). Interestingly, the work described below unexpectedly found that this mutation, S14A, renders cells more resistant to hydrogen peroxide (Figure 5.3b). As a result the effects of hydrogen peroxide on Cdc37 levels were also analysed here.

5.2 Results

5.2.1 Cdc37 is a relatively stable protein whose levels are not strongly affected by altered activity of Ppt1 protein phosphatase or CK2 protein kinase.

The elevated levels of Cdc37 in yeast cells which express the S14A, S14E or S17A mutant versions of this protein (Figure 3.3) might reflect these mutations acting to increase stability of this protein. Alternatively they might be a reflection of increased expression of the *CDC37* gene. Halflives of individual proteins in yeast are often measured as the kinetics of their decline in cells treated with the protein synthesis inhibitor cycloheximide (Belle et al., 2006). We were unable to detect any reduction of Cdc37 level in BY4741 cells treated with cycloheximide. Instead this protein appeared to be even more stable than the actin used as a protein loading control on the blots (Figure 5.1a).

These S14A, S14E and S17A mutant forms of Cdc37 are defective in CK2 phosphorylation/Ppt1 dephosphorylation of this chaperone, important for the ability of Cdc37 to facilitate its activation of nascent protein kinase molecules (Bandhakavi et al., 2003; Mandal et al., 2007; Vaughan et al., 2008). We therefore analysed how Cdc37 level is affected by: (i) the loss of Ppt1 (comparing a *ppt1Δ* delete and the corresponding wild type strain of BY4741 genetic background (Table 2.1); Figure 5.1a); (ii) *PPT1* gene overexpression in cells expressing either the wild type or S14 mutant Cdc37-HA (strains DH211 and DH212 bearing a plasmid with a *MET25* promoter regulated *PPT1* gene; (Figure 5.1b); (iii) the expression of a temperature sensitive *cka2* mutation (*cka2* ^{ts} and wild type W303-based strains (Table 2.1); Figure 5.1c)). In these experiments, either the loss or

overexpression of Ppt1 appeared not to have any appreciable effect on Cdc37 levels (Figure 5.1a,b), whereas *cka2* ^{ts} appeared to be having opposite effects on levels of Cdc37 at 25° and at 37°C (Figure 5.1c).

Taken together these experiments indicate Cdc37 to be a relatively stable protein, whose levels are not subject to rapid change in response to either the inhibition of protein synthesis or alterations to CK2 and Ppt1 activity.

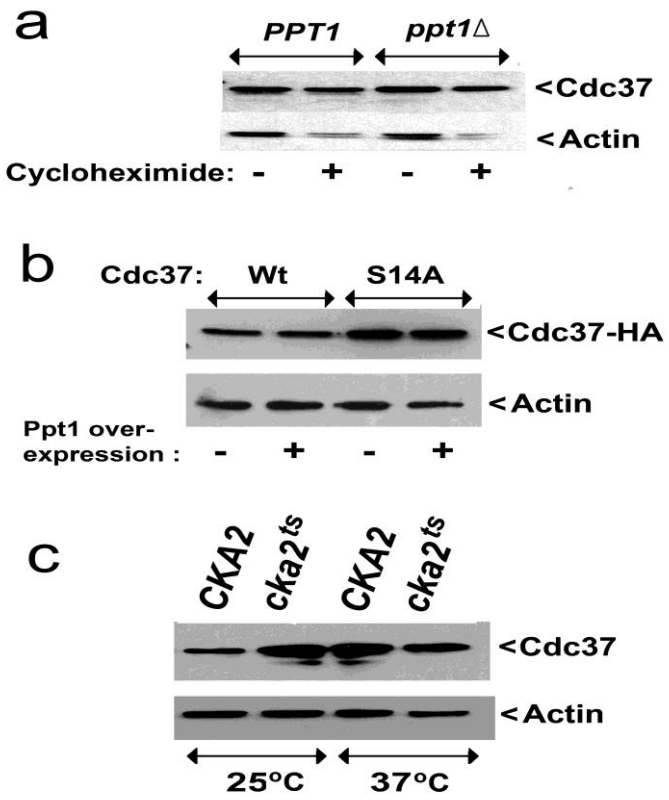


Figure 5.1 Measurement of Cdc37 and actin levels in: (a) Strain BY4741 and an isogenic *ppt1Δ* delete, before and after a 4h 28° treatment with 1mg/ml cycloheximide. (b) Cultures expressing a wild type (Wt) or S14 mutant Cdc37-HA (strains DH211 and DH212) and transformed with a *URA3* vector for *MET25* promoter regulated *PPT1* overexpression, cells either with normal Ppt1 levels (-) or overexpressing Ppt1 (+). (c) A temperature sensitive *cka2* mutant and corresponding wild type (Strains YDH13 (*cka2* ^{ts}) and YDH6 (*CKA2)*; Table 2.1) in the W303 genetic background either maintained at 25° or shifted from 25° to 37°C for 2h.

5.2.2. Cdc37 levels are not responsive to general proteotoxic stress.

We next considered whether the increased Cdc37 level in cells expressing S14A, S14E, S17A mutant Cdc37 might be reflection of proteotoxic stress, due to these cells being deficient in stabilising much of their kinome (Mandal et al., 2007). A number of studies have examined the transcriptional effects of such proteotoxic stress in yeast, a stress often induced in cells through chemical inhibition of the proteasome with MG132 (Zhang et al., 2013). Such inhibition of the proteasome induces proteasomal genes through the stabilization of Rpn4, a transcription factor that is normally short lived as it is itself degraded by the proteasome and whose stabilisation, in response to proteotoxic stress, upregulates the abundance of proteasomal components (Xie and Varshavsky, 2001). However no elevations in Cdc37 level were apparent in wild type (DH211) cells treated with MG132, an inducer of this Rpn4-mediated response (Figure 5.2c). Levels were also unaffected by up to 8h treatment with paromomycin, an antibiotic that generates proteotoxic stress and the induction of the heat shock factor (Hsf1)-mediated heat shock response by causing a high level of translational error (Grant et al., 1989) (Figure 5.2d).

5.2.3 Compromised Hsp90 chaperone activity produces little increase in Cdc37.

Having discounted general proteotoxic stress as the probable cause of the increased Cdc37 level in cells expressing S14A, S14E or S17A mutant Cdc37, we next investigated whether Cdc37 levels are subject to change in response to Hsp90 inhibitors. For this we treated cells with either the selective Hsp90 inhibitor radicicol or (see below) with molybdate. With radicicol treatment cells expressing the wild type Cdc37-HA (strain DH211) displayed little increase in the level of this Cdc37-HA but a strong induction of Hsp90 (Figure 5.2a). This induction of Hsp90 reflects radicicol being a potent inducer of the Hsf1-mediated heat shock response, Hsf1 being the major regulator of the genes for Hsp90 in yeast (Harris et al., 2001;

Piper et al., 2003). However there is still no evidence for Cdc37 being Hsf1 regulated and Cdc37 levels are only modestly, if at all, affected by the Hsf1 inducers radicicol (Figure 5.2a) or paromomycin (Figure 5.2d).

5.2.4 *In vivo* molybdate treatment results in the loss of Cdc37, as well as the appreciable fragmentation of Hsp90.

Molybdate is often added to mammalian cell extracts in order to stabilise the complexes forming between Hsp90 and its "client" proteins (see (Soti et al., 1998) for literature). It has been proposed that the MoO_4^{2+} oxyanion, through acting as a transition state analogue of phosphate inhibits the final, essential ATPase step of the Hsp90 chaperone cycle and the associated, p23-cochaperone assisted release of the activated client protein (Hartson et al., 1999; Pratt and Toft, 1997; Young and Hartl, 2000). Hsp90: client complexes are thereby arrested in a p23-bound state, in which the client protein is prevented from achieving its final active form (see Discussion).

This action of molybdate is strikingly different from that of the Hsp90 inhibitor antibiotic radicicol. While geldanamycin, radicicol and molybdate both bind Hsp90 directly (Prodromou et al., 1997; Roe et al., 1999; Soti et al., 1998; Stebbins et al., 1997), they exert opposing effects on Hsp90-client protein interactions. Geldanamycin and radicicol, inhibitors of the ATP binding step (Prodromou et al., 1997; Roe et al., 1999), induce an ADP/ATP-free state of the chaperone that has low affinity for the client substrate. This leads to a marked destabilisation of Hsp90 client proteins *in vivo* (Blagosklonny et al., 1996; Maloney and Workman, 2002; Ochel et al., 2001; Smith et al., 1998; Whitesell et al., 1997). Molybdate, in contrast, is thought to inhibit at a later stage in the chaperone cycle when the client protein is much more tightly bound to the Hsp90 machine; inducing a "locked" form of the client-chaperone complex in which the client is stabilised through strong hydrophobic

interactions with the ADP or ATP-bound state of the chaperone (Hartson et al., 1999; Pratt and Toft, 1997). Thus geldanamycin destabilises client proteins *in vivo* whereas molybdate – at least *in vitro* - exerts the opposite effect, stabilising Hsp90-client interactions.

The growth inhibitory effects of high molybdate levels on yeast cells have been shown to arise mainly from effects on the Hsp90 chaperone system (Millson et al., 2009). When DH211 yeast cells were treated for 2h and 4h with 20mg/ml sodium molybdate, a concentration shown as inhibitory to the growth of this strain (Millson et al., 2009), we unexpectedly observed a marked destabilisation of Cdc37-HA, p23 and Hsp90 (Figure 5.2a). Notably - after 4h at 28°C in the presence of molybdate - the Cdc37 and Hsp90 of these cells had almost totally lost its integrity, much of the latter chaperone degrading to a discrete 65kDa subfragment of Hsp90 (**; Figure 5.2a-c). Using cells with a wild type or S14A mutant Cdc37-HA (DH211/2) transformed with a vector for Slt2-HA expression, we were able to show that this molybdate treatment also results in the loss of a Cdc37/Hsp90 client, the MAP kinase Slt2(Mpk1) (Hawle et al., 2007; Millson et al., 2005) (Figure 5.2b).

This molybdate destabilisation of Cdc37 and Hsp90 in DH211 cells was not appreciably slowed by the presence of the proteasome inhibitor MG132 (Figure 5.2c). We subsequently found that it was more marked when cells were maintained at 37°C as compared to 28°C, so many of our later molybdate experiments (see below) involved either a 2h or 4h treatment with 10mM molybdate at 37°C rather than a 2h treatment with 20mM molybdate at 28°C.

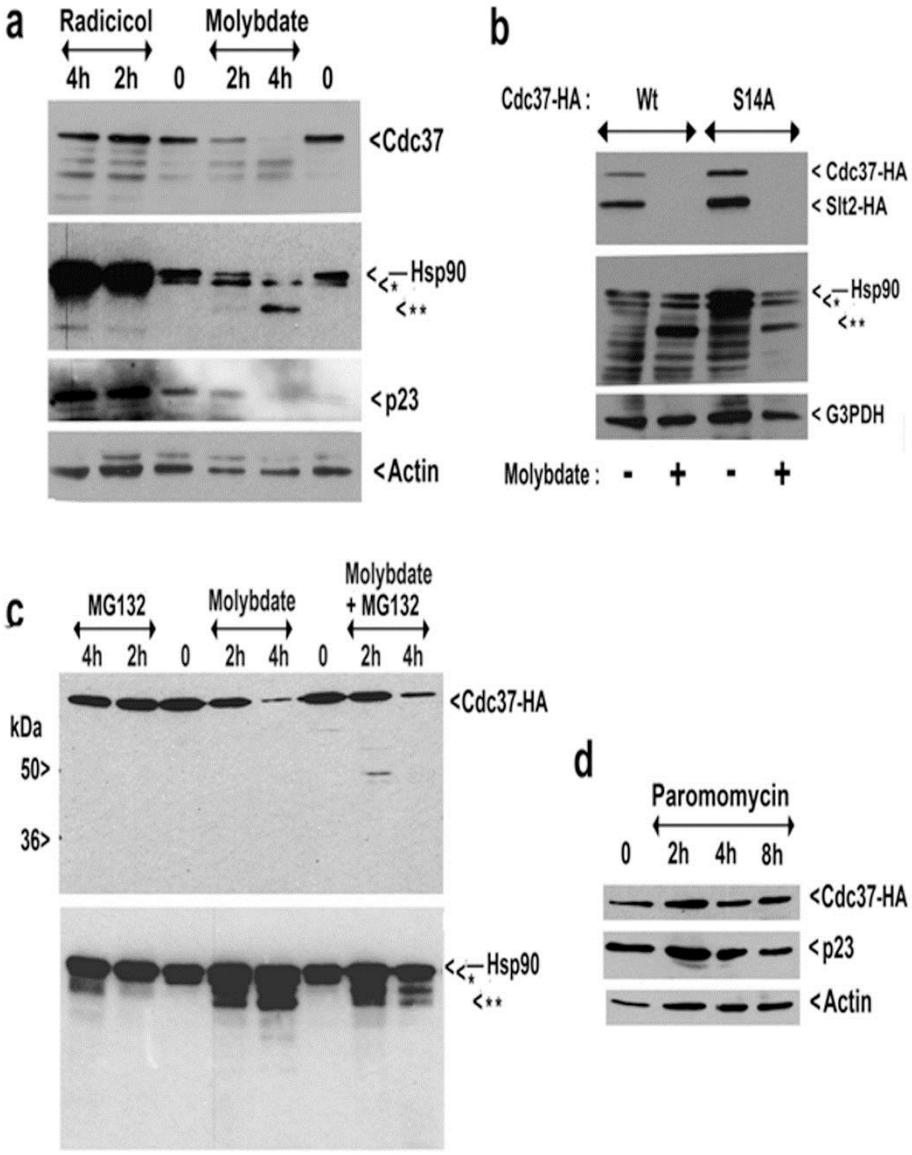


Figure 5.2 (a) Analysis of Cdc37, Hsp90, p23 and actin levels in DH211 cells before (0) or 2h, 4h following treatment at 28°C with either 60µg/ml radicicol or 20mg/ml sodium molybdate. *, ** indicate respectively the major fragment of Hsp90 resulting from oxidative free radical damage oxidative damage (Beck et al., 2012) (*) and the Hsp90 fragment that accumulates in response to molybdate-treatment (this study) (**). (b) Analysis of Cdc37-HA, Slt2-HA, Hsp90 and G3PDH levels in DH211/2 cells containing a vector for Slt2-HA expression before (-) or 4h following (+) molybdate treatment. (c)Analysis of the effects of the proteasome inhibitor MG132, an inducer of the Rpn4-mediated response to proteotoxic stress, on the Cdc37-HA and Hsp90 levels of DH211 cells both with and without molybdate under the conditions in (a). (d) Analysis of the effects of paromomycin (1mg/ml), an antibiotic that generates proteotoxic stress by causing a high level of translational error, on the Cdc37-HA and p23 levels of DH211.

5.2.5. Cdc37 is a determinant of oxidative stress resistance in yeast.

N-terminally truncated forms of Hsp90 are known to accumulate *in vivo* (Panaretou et al., 1999) and, in mammalian cells, these have been shown to result from oxidative damage (Beck et al., 2012). This raised the possibility that the loss of Cdc37 and fragmentation of Hsp90 we had observed in molybdate-treated cells (Figure 5.2) was the result of damage to these chaperone molecules caused by oxygen free radicals. Consistent with this is the observation that these events still occurred in the presence of the proteasome inhibitor MG132 (Figure 5.2c).

Analysing the resistance of our strains to hydrogen peroxide (H_2O_2) we noted that resistance to this oxidant was increased with overexpression of the wild type Cdc37 protein (Figure 5.3a). Remarkably, 28°C oxidant resistance was also increased in the cells expressing the S14A mutant form of Cdc37 (Figure 5.3b), despite this being a mutation that sensitises cells to high temperature stress and the loss of Hsp90 activity (Bandhakavi et al., 2003; Vaughan et al., 2008). As Cdc37 had not previously been identified as a determinant of oxidant resistance we next analysed whether H_2O_2 stress affects cellular levels of Cdc37.

Analysing the effects of H_2O_2 treatment on the Cdc37-HA and Hsp90of DH211 cells, we found that a H_2O_2 concentration which causes appreciable loss of viability in plate assays (Figure 5.3) also resulted in appreciable loss of Cdc37 protein at 37°C but not 28°C, this effect being reduced slightly by a simultaneous treatment with the proteasome inhibitor MG132 (Figure 5.4a). It is known that the effects of oxidative stress are generally greater at higher temperatures. Further evidence that this reduction in Cdc37 level with H_2O_2 treatment is a consequence of oxidative stress came from an analysis of the *ctt1* mutant, lacking the major cytosolic catalase of yeast. When exposed to H_2O_2 – though not in this particular

experiment molybdate - the *ctt1* mutant displayed a greater loss of Cdc37 relative to the corresponding wild type (Figure 5.4b).

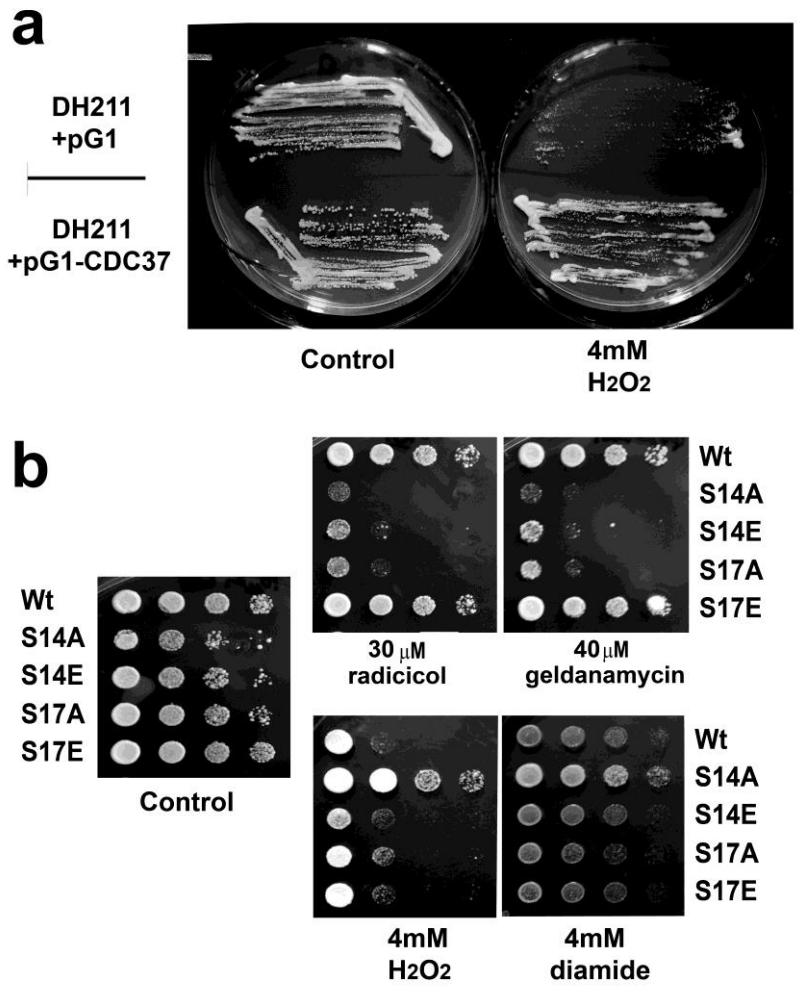


Figure 5.3 (a) Analysis of the resistance to H_2O_2 in DH211 cells bearing either empty pG1 vector or pG1-CDC37 (the latter a vector for GPD1-promoter driven Cdc37 overexpression). Cells were streaked on minus Trp SD medium either with or without 4mM H_2O_2 and the plates photographed after 3d at 28°C. (b) A comparative analysis of the resistances of strains DH211-5 to Hsp90 inhibitor antibiotics (radicicol, geldanamycin) and oxidants (H_2O_2, diamide). Serial dilutions of YPD grown cultures were pinned onto YPD agar containing the indicated chemical and plates photographed after 2d at 28°C.

5.2.6 The effects of hydrogen peroxide indicate that it is the MoO_4^{2+} oxyanion, not peroxomolybdate, which destabilises Cdc37 and Hsp90 *in vivo.*

In the presence of H_2O_2 the molybdate anion is converted to diperoxomolybdate, a sulphydryl (–SH) selective reagent (Mikalsen and Kaalhus, 1997). The *in vitro* effects of molybdate on mammalian Hsp90, especially in the presence of H_2O_2, have earler been attributed to diperoxomolybdate labelling of the –SH groups that exist primarily in the middle and C-terminal regions of this chaperone (Soti et al., 1998). Such *in vitro* diperoxomolybdate labelling of purified mammalian Hsp90 protein is unaffected in the presence or absence of ADP or ATP and results in a characteristic shift in gel mobility which is, in turn, reversed by dithiothreitol treatment (Soti et al., 1998). However, unlike the mammalian Hsp90, the yeast Hsp90 and Cdc37 proteins analysed in this study have no free –SH groups. The molybdate-induced loss of Cdc37 and fragmentation of Hsp90 that we had observed occurring in yeast (Figure 5.2) cannot therefore be attributed to direct diperoxomolybdate modification of –SH groups on either Cdc37 or Hsp90.

We noted that H_2O_2 strongly inhibited this molybdate-induced loss of Cdc37 and conversion of much of the cellular Hsp90 to a discrete 65kDa subfragment *in vivo* (Figure 5.4c). That the presence of H_2O_2, leading to the conversion of much of the molybdate to diperoxomolybdate, potently abolishes this molybdate-induced destabilisation of Cdc37 and fragmentation of Hsp90 is a strong indication that these events are the result, not of diperoxomolybdate reaction with components of the Hsp90 chaperone system, but a more direct action of the MoO_4^{2+} oxyanion.

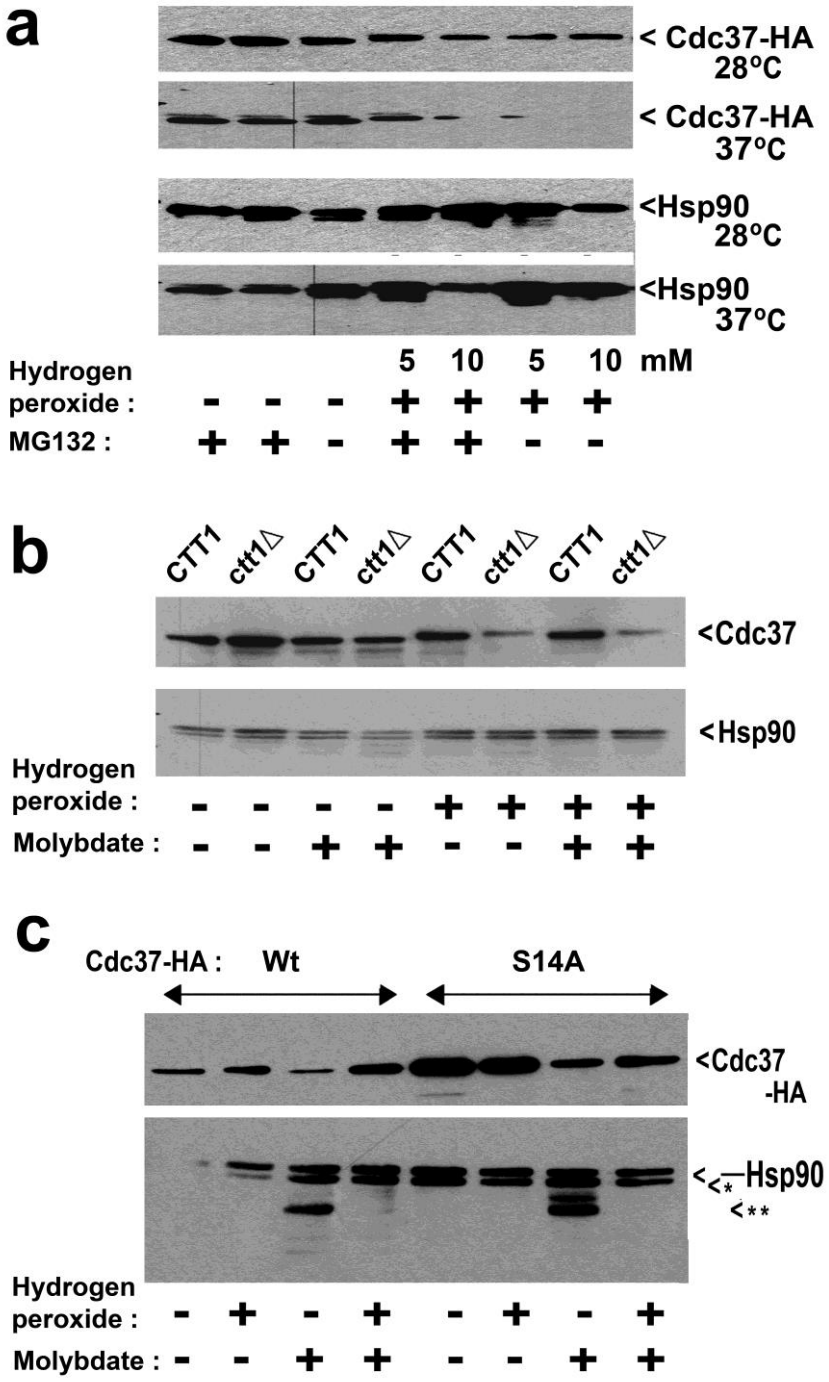


Figure 5.4 (a) Analysis of the effects of a 5 or 10mM H_2O_2 treatment for 2h at either 28°C or 37°C on the Cdc37-HA and Hsp90 levels of DH211 cells. (b) Analysis of the effects of a 2h 28°C 10mM molybdate and/or 20mM H_2O_2 treatment on Cdc37 and Hsp90 levels in a *ctt1* mutant and its corresponding FY1679 wild type. (c). Analysis of the effects of a 2h 37°C 10mM molybdate and/or 20mM H_2O_2 treatment on Cdc37-HA and Hsp90 levels in DH211 and DH212 cells. *, ** indicate respectively the major fragment of Hsp90 resulting from oxidative free radical damage oxidative damage (Beck et al., 2012) (*) and the major fragment of Hsp90 accumulating following molybdate-treatment (**).

5.2.7 Radicicol reduces the loss of Cdc37, but not the fragmentation of Hsp90 *in vivo* in molybdate treated cells.

In vitro, molybdate has been shown to inhibit the Hsp90-dependent biogenesis of $p56^{lck}$F505 in rabbit reticulocyte lysate by physically locking $p56^{lck}$F505 onto the Hsp90 machinery, the normally salt-labile interaction of Hsp90 with heat-denatured luciferase being rendered salt-resistant by molybdate. An addition of geldanamycin prior to molybdate prevented this molybdate-induced salt resistance whereas with the reverse order of addition of these reagents (adding molybdate before geldanamycin), geldanamycin did not compromise molybdate-induced salt resistance (Hartson et al., 1999). Thus the order of addition of an Hsp90 inhibitor and molybdate can affect the outcome of experiments using these two agents (Hartson et al., 1999; Pratt and Toft, 1997). However a pretreatment of rabbit reticulocyte lysate with 20 mM molybdate was found to inhibit the subsequent ability of Hsp90 to bind immobilised geldanamycin (Hartson et al., 1999).

If molybdate causing loss of Cdc37 and the fragmentation of much of the cellular Hsp90 in yeast (Figures 5.2; 5.4c) reflects a selective binding of the MoO_4^{2+} oxyanion to just the ADP or ATP-bound states of the chaperone, these processes should be slowed when radicicol occupies the ADP/ATP site on Hsp90, thus preventing binding of ADP/ATP to this site. However only a moderate reduction in molybdate-induced loss of Cdc37-HA was observed when a growth inhibitory level of radicicol was added to DH211 cells 10min before, or at the same time as, the addition of molybdate. Molybdate treatment still caused a substantial reduction in Cdc37-HA levels under these conditions (Figure 5.5a,b). In contrast no such partial rescue of Cdc37 loss was apparent when the radicicol was added to the cells 10min after they had been treated with molybdate (Figure 5.5a). Molybdate also caused an appreciable Cdc37-HA loss in *(S14A)cdc37* cells (Figures 5.4c; 5.5b) and, while it did appear

that the levels of Cdc37 degradation might be reduced in this mutant as compared to the wild type (Figures 5.4c; 5.5b), this might be merely reflect the higher initial levels of Cdc37-HA in these *(S14A)cdc37* cells (Figure 3.3).

Importantly, and in marked contrast to Cdc37, the molybdate degradation of Hsp90 did not appear to be affected by the presence of radicicol in any of these experiments (Figure 5.5a,b). Since molybdate-induced loss of Cdc37 is partially abrogated by radicicol (Figure 5.5a), this loss is – at least to a limited degree – Hsp90-dependent and most probably therefore a consequence of Hsp90 binding the MoO_4^{2+} oxyanion. The binding of ATP/ADP to the N-terminal domain of Hsp90 is known to be prevented as the middle region of Cdc37 binds this Hsp90 domain causing a transient arrest of the Hsp90 chaperone cycle (Roe et al., 2004; Siligardi et al., 2002). On the basis of current evidence the bindings of either radicicol or Cdc37 at this region of Hsp90 appear therefore to be mutually exclusive.

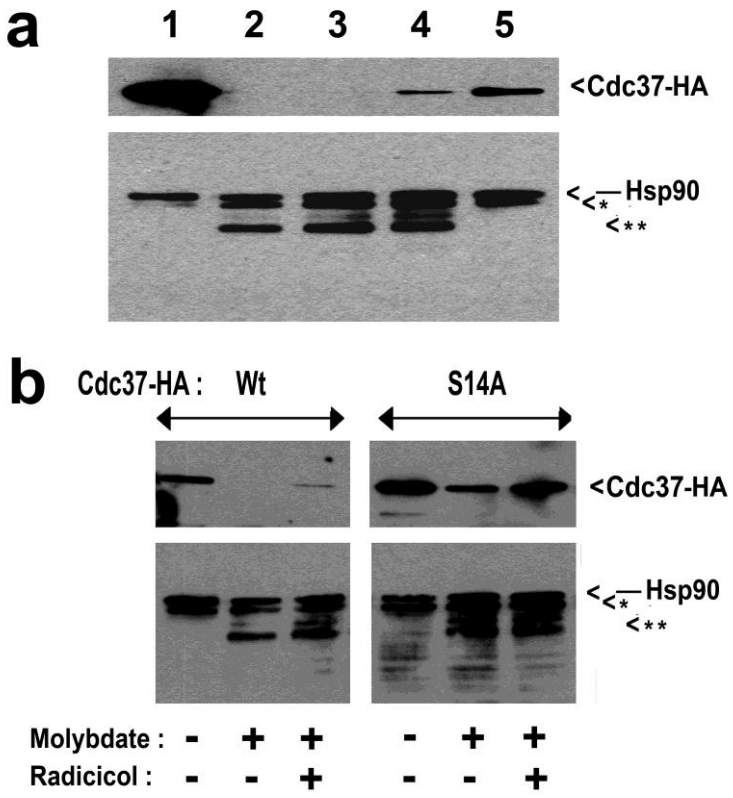


Figure 5.5 Analysis of the effects of a growth inhibitory level of radicicol on the molybdate-induced degradation of Cdc37-HA and Hsp90. (a) DH211 cells growing at 37°C were exposed to either: (1) no treatment, (2) 10mM molybdate 2h 37°C, (3) 10mM molybdate for 10min, followed by the addition of 50µM radicicol and the cells maintained a further 2h at 37°C; (4) 50µM radicicol for 10min, then 10mM molybdate added and the cells maintained a further 2h at 37°C; (5) 50µM radicicol 2h 37°C. (b) An analysis of the effects of radicicol (100µg/ml) on molybdate-induced degradation of Cdc37-HA and Hsp90 in cells expressing either wild type (wt; DH211) or S14A mutant (DH212) Cdc37-HA. Radicicol and/or molybdate were added simultaneously to 28°C cultures, cells then being maintained 2h at 37°C. Cdc37 was analysed using an anti-HA antiserum. *, ** indicate respectively the major fragment of Hsp90 resulting from oxidative free radical damage oxidative damage (Beck et al., 2012) (*) and the major fragment of Hsp90 accumulating following molybdate-treatment (**).

5.2.8 The human Hsp90α expressed in yeast also displays molybdate-induced fragmentation.

The above experiments describe how an *in vivo* treatment of yeast cells with high levels of molybdate causes loss of Cdc37 and the conversion of much of the cellular Hsp90 to a discrete 65kDa subfragment. There is currently considerable interest in drug therapies that can inactivate Cdc37 (Pearl, 2005; Smith and Workman, 2009) or Hsp90 (Neckers and Workman, 2012; Trepel et al., 2010), since these are chaperones vital for the activity of several of the oncoprotein "drivers" of cancer. Whilst it will be important to know if molybdate treatment could inactivate the Cdc37 and Hsp90 in cancer cells, as well as in yeast, such cancer cell studies are outside the scope of the analysis performed in this thesis. While mammalian Cdc37 expression cannot provide the Cdc37 function in yeast, the human cytosolic Hsp90s are functional in this model eukaryote (Millson et al., 2007). We therefore compared the effects of molybdate on yeast strains expressing either the native yeast Hsp82 or the human Hsp90α as their sole Hsp90. This revealed the human Hsp90α expressed in yeast also accumulated as a 65kDa subfragment when cells were treated with molybdate (Figure 5.6).

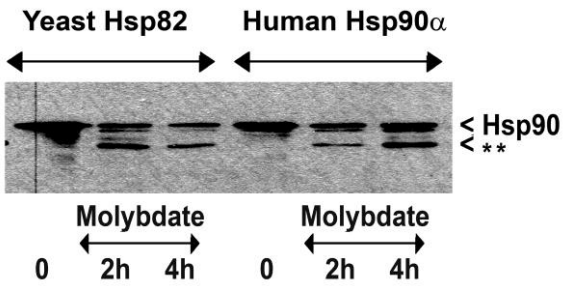


Figure 5.6 Analysis of Hsp90 in cells of the PP30 genetic background expressing either the native yeast Hsp82 or the human Hsp90α as their sole Hsp90 (PP30-HSP82 and PP30-huHSP90α, Table 2.1) treated with 10mM molybdate 2h or 4h at 37°C. The major fragment of Hsp90 accumulating following molybdate-treatment is indicated (**).

5.3 Discussion

This study was initiated in order to try to understand why yeast cells expressing the S14A, S14E or S17A mutant versions of Cdc37 have higher levels of this cochaperone protein (Figure 3.3). While it did not succeed in showing why these mutations have this effect, it did reveal Cdc37 to be normally a stable protein (Figure 5.1) and also yield two quite unexpected findings: firstly, that Cdc37 and Hsp90 are rendered unstable in molybdate treated cells (Figure 5.2) and, secondly, that Cdc37 can be a determinant of oxidative stress resistance, as well as a target of oxidative stress (Figures 5.3, 5.4).

Molybdate is often added to cell extracts in order to stabilise the complexes forming between Hsp90 and its "client" proteins (see (Soti et al., 1998) for literature). The transition metal oxyanions molybdate, vanadate and tungstate have all been found to stabilise the presence of Cdc37(p50) in the $pp60^{v\text{-}src}$ –Hsp90 heterocomplex (Hutchison et al., 1992). Molybdate stabilises the normally salt-labile interaction of steroid receptors with Hsp90, p23 and immunophilins, but not the heterocomplexes between these receptors, Hop and Hsp70 (Chen et al., 1996). It has also been shown to inhibit Hsp90-mediated biogenesis of the $p56^{lck}$ protein kinase and to render the Hsp90 interaction with this kinase salt stable (Hartson et al., 1999). While it decreases Hsp90's ability to bind geldanamycin, it stabilises the association of Hsp90 with p23 (Hartson et al., 1999). This led Toft and co-workers to propose that ADP-MoO_4^{2+} was replacing ATP in these molybdate-inhibited p23-comtaining complexes (Pratt and Toft, 1997).

The novelty of the work in this chapter is that it shows for the first time that an *in vivo* treatment of cells with millimolar levels of molybdate leads to both the loss of Cdc37 and a discrete fragmentation event within the Hsp90 molecule. This molybdate-induced fragmentation of Hsp90 and destabilisation of Cdc37 are not the result of a

diperoxomolybdate reaction with –SH groups on these chaperones (Figure 5.4c), and therefore most probably reflect actions of the MoO_4^{2+} oxyanion. The Hsp90 fragmentation involves the cleavage of a specific peptide bond within this chaperone, a reaction that *in vivo* appears largely unaffected by the Hsp90 inhibitor radicicol (Figure 5.5). This cleavage still occurs with the purified yeast Hsp82 protein, prepared following expression in recombinant *E. coli*, incubated with molybdate (not shown). It appears therefore that it most probably reflects a chemical reaction catalysed by the Hsp90-bound MoO_4^{2+} oxyanion.

Chapter 6: Significance of this thesis study and the possibilities for future work.

This study has uncovered a number of novel findings and highlighted a number of aspects that need further investigation:

The work in Chapter 3 revealed for the first time that cells expressing S14A, S14E, S17A mutant versions of Cdc37 have elevated levels of Cdc37. It confirmed Cak1 forms a stable association with Cdc37, reinforced by the S14A mutation. However, despite much of the yeast kinome being destabilised in *cdc37(S14A)* cells (Mandal et al., 2007), Cak1 expression levels appear unaffected by this mutation (Figure 3.3). Even though Cak1 appears not to require the CK2 phosphorylation/Ppt1 dephosphorylation of Cdc37 either for its activity or stability, events so vital for stabilization of much of the kinome, one must ask why it forms a relatively stable complex with Cdc37. Is this association perhaps facilitating Cdc37 association with Cdc28, the latter an activity destabilised in *cdc37(S14A)* cells and activated by Cak1?

Chapter 3 also identified an apparent change to the phosphorylation status of the Cak1 in Cdc37(S14A) association (Figure 3.5). Two new sites of Cak1 phosphorylation, Thr 27 and Ser 172, were identified (Figure 4.1). Versions of strain SY80 were made in which the sole Cak1 is a form in which each of these residues is either a nonphosphorylatable alanine, or a phosphomimetic residue (Chapter 4). While these strains exhibited some marked phenotypes (Figures 4.2 and 4.3), they are almost certainly massively overexpressing the relevant point mutant form of Cak1. Not only might this have affected the phenotypes observed, but also – through titration effects – it might influence the results of pull-down experiments showing

how these mutations influence the protein associations of Cak1. Probably therefore the analysis in Chapter 4 should be extended to cells in which these mutant forms of Cak1 are expressed from the native *CAK1* gene promoter. Time constraints did not allow this to be done. Neither did it allow us to analyse how these phosphorylation site mutations affect the formation of the Cak1/Cdc37 complex identified in Chapter 3. Furthermore, as Ser 172 is within a Ser-Pro motif, it might possibly be executed by Cdc28. Analysis of its phosphorylation status in a Cdc28-analogue sensitive yeast strain (Knight and Shokat, 2007) might reveal if this is the case and reflects a hitherto unidentified Cdc28 control over its regulator Cak1.

Chapter 5 shows for the first time that an *in vivo* treatment of yeast with millimolar levels of molybdate leads to both the loss of Cdc37 and a discrete fragmentation event within the Hsp90 molecule. High molybdate levels must therefore be causing a total inactivation of these essential chaperone functions in yeast cells, probably a major reason why these levels of molybdate result in loss of viability (Millson et al., 2009). Molybdate may also cause loss of other Hsp90 system cochaperones besides Cdc37 (indeed we obtained initial data indicating it was also resulting in the loss of p23; see Figure 5.2a). However time did not allow these other cochaperones to be studied.

How does molybdate cause loss of Cdc37 (Figure 5.2)? Does it reflect binding of the MoO_4^{2+} oxyanion to intact Hsp90, an action due to the MoO_4^{2+} -cleaved 65kDa fragment of Hsp90 or does this Cdc37 loss occur largely independently of Hsp90? Here we only found partial rescue of Cdc37 loss with use of the selective Hsp90 inhibitor radicicol and then only when the radicicol was added prior to the addition of molybdate (Figure 5.5).

This molybdate-induced fragmentation of Hsp90 and destabilisation of Cdc37 are not the result of a diperoxomolybdate reaction with –SH groups on these chaperones (Figure 5.4c),

and therefore most probably reflect actions of the MoO_4^{2+} oxyanion. The Hsp90 fragmentation involves the cleavage of a specific peptide bond within this chaperone, a reaction that *in vivo* appears largely unaffected by the Hsp90 inhibitor radicicol (Figure 5.5). It is now important to identify, by mass spectrometry, the site of this cleavage in Hsp90. Also does it still occur when the purified yeast Hsp82 protein, prepared following expression in recombinant *E. coli*, incubated with molybdate. If so it most probably reflects a chemical reaction catalysed by the Hsp90-bound MoO_4^{2+} oxyanion.

Another issue raised by the studies in Chapter 5 is why is Cdc37 a determinant of oxidative stress resistance (Figure 5.3)? Is it because key components needed for oxidative stress signalling are Cdc37-dependent? However the Hog1 MAP kinase, key for such signalling, (Proft and Struhl, 2004), is compromised in its function by the *cdc37(S14A)* mutation (Hawle et al., 2007), a mutation that we found actually enhances H_2O_2 resistance (Figure 5.3). Clearly this requires further study.

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