SRICHARAN BANDHAKAVI
Protein kinase CKII and Cdc37 constitute a positive feedback loop that maintains multiple protein kinases
(Under the direction of CLAIBORNE V. C. GLOVER III)

Cdc37 is a protein kinase-specific chaperone that either acts by itself or in concert with Hsp90. Cdc37 is phosphorylated by the evolutionarily conserved, ubiquitously distributed Ser/Thr kinase, CKII; and this dissertation describes efforts to analyze the importance of Cdc37 phosphorylation by CKII and its potential role in regulating Cdc37 function *in vivo*. Non-phosphorylatable (*cdc37-S14AS17A*), semi-phosphorylatable (*cdc37-S14A)*, *cdc37-S17A*), and quasi-phosphorylated (*cdc37-S14ES17E*) alleles of *CDC37* were constructed and characterized. *cdc37-S14AS17A* was the most severely affected of all alleles and displayed a semi-lethal phenotype underscoring the importance of the CKII phosphorylation sites. *cdc37-S14A* was more severely affected than *cdc37-S17A* mutants, supporting the idea that the evolutionarily conserved CKII site at serine-14 is more important in regulating Cdc37 function than serine-17. *cdc37-S14A* arrested with reduced CKII activity, and overexpression plasmids expressing *cdc37-S14AS17A* or *cdc37M2-S14A* were incapable of protecting *cka2-13* mutants on media containing Geldanamycin (GA), an Hsp90-specific inhibitor. Incubation of *cka2-13* cells with GA results in reduced phosphorylation of Fpr3 by CKII. Additionally, CKII activity was found to peak during G1 and G2/M phases of the cell cycle, the same phases during which Cdc37 function is essential. Genetic assays were used to demonstrate that the positive feedback loop between CKII and Cdc37 also maintains the activity of multiple protein kinases (and hence their signal transduction pathways), in addition to CKII. Overexpression of the homologous gene pair, *ZDS1/2* was found to augment the function

of both CKII and Cdc37, thus offering a potential mechanism for the observed pleiotropism of Zds1/2 since the feedback loop between CKII and Cdc37 regulates diverse pathways.

INDEX WORDS: CKII, Cdc37, Hsp90, Zds1/2

PROTEIN KINASE CKII AND *CDC37* CONSTITUTE A POSITIVE FEEDBACK LOOP THAT MAINTAINS MULTIPLE PROTEIN KINASES

by

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DEDICATION

To Bammagaru, Mom and Dad

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Protein Kinases

Many cellular functions are regulated by post-translational modification of the proteins that perform them. There are more than 20 post-translational modifications that occur on eukaryotic proteins (Parekh and Rohlff, 1997). Several of these modifications, with phosphorylation being the paradigm, participate in diverse signal transduction pathways and cellular/organismal responses to the environment. Protein kinases, the enzymes that catalyze the transfer of the phosphoryl group to target proteins (substrates), play a major role in several processes that make life possible.

Protein kinases are mainly classified based on the amino acid residues phosphorylated by them. Prokaryotic kinases include histidine/arginine/lysine kinases (E.C. 2.7.12), cysteine kinases (E.C. 2.7.13), and aspartyl/glutamyl kinases (E.C. 2.7.14). Eukaryotic kinases include, serine/threonine kinases (E.C. 2.7.10) or tyrosine kinases (E.C. 2.7.11). The genome of *Saccharomyces cerevisiae* is predicted to encode 113 potential protein kinases (Hunter and Plowman, 1997), and estimates of the number of protein kinases in mammalian systems hover close to 2000 (Hunter, 1994). The protein kinase group is the second largest group of genes in the eukaryotic genome, second only to transcription factors.

Although protein kinases can be very divergent in terms of amino acid composition, the catalytic core of all eukaryotic kinases is topologically similar (Johnson et al., 1996). Conserved features have been identified in each of the 12 subdomains common to these protein kinases, and residues from these subdomains play essential roles in kinase structure and function. The kinase core consists of a bilobed scaffold that has a smaller N-terminal lobe composed almost entirely of antiparallel β sheets and a C-terminal lobe rich in α helices. The catalytic site, located at the interface of the two lobes, is the site of phosphotransfer from ATP (or GTP) to target residues (Johnson et al., 1996).

Substrate specificity of protein kinases is determined by a combination of the 3-D structure of the kinase and the structure/sequence composition of the substrate. The few amino acid residues adjacent to the Ser/Thr/Tyr site being phosphorylated are important determining factors for the phosphorylation event. For example, protein kinase CKII phosphorylates Ser/Thr/Tyr residues in an acidic context (Guerra and Issinger, 1999), while CDK2 phosphorylates ser/thr residues followed by a proline (Johnson et al., 1996). Regulatory subunits of protein kinases can stimulate/inhibit the phosphorylation event depending on the particular kinase/substrate in question. For example, the CKIIβ subunit stimulates CKII activity toward most substrates but inhibits the phosphorylation of calmodulin by CKII (Bidwai et al., 1993).

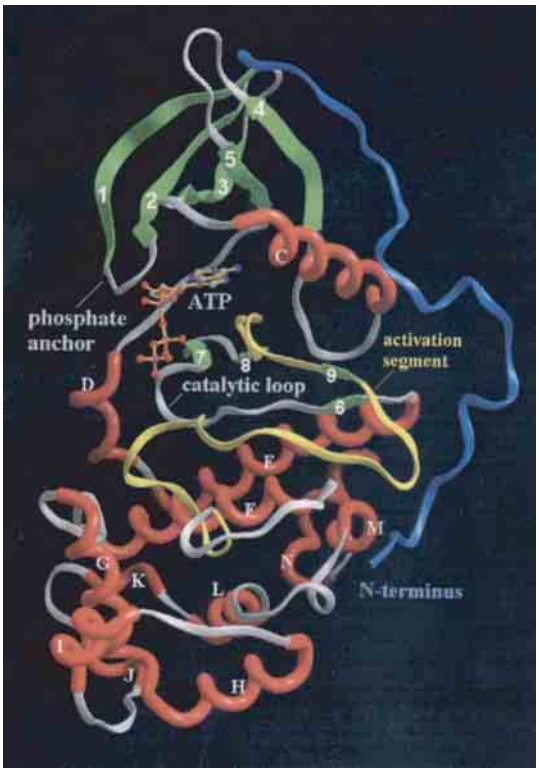
Protein Kinase CKII

Protein Kinase CKII (or Casein Kinase II) is a ubiquitous Ser/Thr/Tyr kinase expressed in all eukaryotes. From most sources, the enzyme is composed of two

polypeptide subunits, α(35-44 KDa) and β(24-28 KDa), which combine to form an $\alpha_2\beta_2$ tetramer (for a review see Allende and Allende, 1995; Glover, 1998; Guerra and Issinger, 1999; Pinna and Meggio, 1997). In *Saccharomyces cerevisisae*, the enzyme consists of two distinct catalytic subunits, α and α' (encoded by the genes *CKA1* and *CKA2*, respectively), and two regulatory subunits, β and β' (encoded by the genes *CKB1* and *CKB2*, respectively). CKII recognizes Ser/Thr (or in exceptional cases Tyr; see Wilson et al., 1997) in an acidic environment (Guerra and Issinger, 1999). CKII is an essential enzyme in yeast and probably also in *Schizosaccharomyces pombe* (Snell and Nurse, 1994) and *Dictyostelium discoideum* (Kikkawa et al., 1992).

Crystal structures of recombinant maize CKIIα (Niefind et al., 1998), recombinant C-terminally truncated human CKIIβ dimer (Chantalat et al., 1999), and the human CKII tetramer (Niefind et al., 2001) have been solved. The CKII catalytic subunit (Fig. 1.1) consists of a bi-lobed, catalytic core that is typical of protein kinases, and a unique N-terminal segment. The association of the N-terminal segment of the α subunit (approximately 40 residues) with the activation loop (Fig. 1.1) results in the catalytic cleft being open constitutively (Niefind et al., 2001; Niefind et al., 1998). In contrast, the activation loop of cyclin-dependent-kinases (CDKs) is folded back on the catalytic cleft, and the association of cyclin with CDK activates the kinase by pulling the activation segment away from the catalytic cleft. Thus in CKII, the N-terminal segment helps achieve a "constitutively active" state of the enzyme. CKIIβ molecules consist of an N-terminal autophosphorylation site, an internal acidic region, and a zinc-finger motif. The two regulatory subunits form a stable dimer through the zinc finger domain and link with

Figure 1.1. Ribbon diagram of the crystal structure of recombinant maize CKIIα subunit showing interaction regions between the N-terminal segment (blue) and the activation segment (yellow). The position of the active site/catalytic cleft is marked by the bound ATP molecule. This image is taken from Niefind et al. (Niefind et al., 1998).


1
2
3
4
5
C
ATP
phosphate
anchor
activation
segment
7
8
9
D
catalytic loop
6
E
F
M
G
K
L
N
N-terminus
I
J
H

the N-terminal lobes of the two catalytic subunits; the catalytic subunits make no contact with one another (Fig. 1.2) (Niefind et al., 2001).

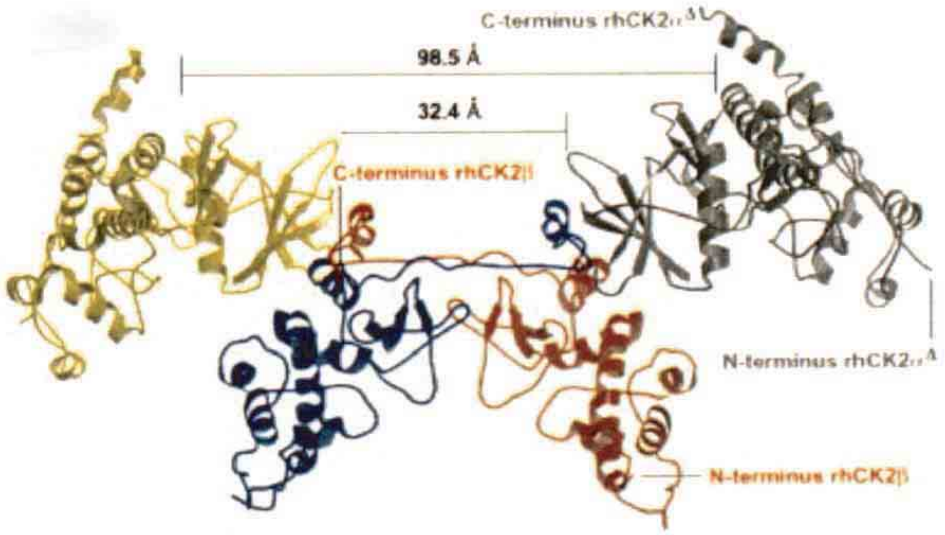
In vitro, the enzyme is inhibited by polyanions such as heparin and stimulated by polycations such as spermine or polylysine. Searches for physiological regulators other than the regulatory subunits of CKII have remained unsuccessful. The basis for activation of CKII activity by the β subunits remains unclear since the association between the N-terminal segment and the activation segment remains unperturbed by β subunits (Niefind et al., 2001). Based on molecular and crystallographic analysis, autophosphorylation of the β subunits is not expected to have any effects on CKII activity either (Niefind et al., 2001). It has been speculated that the β subunits might serve as an environment- and/or substrate-specific modulator of CKII instead.

Physiological Role of CKII

Although CKII was perhaps the first known protein kinase(Dobrowolska et al., 1999), dissecting its precise role has been complicated by the observation that CKII phosphorylates a plethora of substrates involved in diverse cellular functions and little is known about how the enzyme is regulated (Dobrowolska et al., 1999). Known CKII substrates include a broad spectrum of proteins involved in transcription, translation, signal transduction, protein folding, apoptosis, cell cycle regulation, etc. (Guerra and Issinger, 1999).

CKII is probably expressed in all metazoan tissues, but higher activity of CKII is found in dividing cells, both normal and transformed (Issinger, 1993). For example, CKII expression is increased in proliferating cells of the early mouse embryo, in

Figure 1.2. Ribbon diagram of the crystal structure of recombinant human CKII tetramer. The two β subunits (in red and blue) form a dimer, and the two catalytic subunits (in grey and yellow) are not in direct contact with one another. This figure was taken from Niefind et al. (Niefind et al., 2001).


C-terminus rhCK2α'
98.5 Å
32.4 Å
C-terminus rhCK2β
N-terminus rhCK2α'
N-terminus rhCK2β

transformed human cell lines, and in solid human tumors. These observations suggest a positive role for CKII in cellular proliferation. Although CKII is present in both the nucleus and the cytoplasm, the majority of the enzyme is nuclear (Chester et al., 1995; Maridor et al., 1991)

Molecular genetic approaches have been used to explore the physiological role of CKII in multiple organisms/systems (Hanna et al., 1995; Lorenz et al., 1994; Padmanabha et al., 1990; Pepperkok et al., 1994; Rethinaswamy et al., 1998; Roussou and Draetta, 1994; Seldin and Leder, 1995; Snell and Nurse, 1994; Ulloa et al., 1993). In *S. cerevisiae*, deletion of either *CKA1* or *CKA2* alone results in no obvious phenotype on normal media, but deletion of both is lethal, thus demonstrating that CKII is an essential enzyme in *S. cerevisiae* (Padmanabha et al., 1990). CKII is conserved over large evolutionary distances since the inviability of *cka1Δcka2Δ* mutants is rescued by overexpression of the Drosophila α subunit (Padmanabha et al., 1990). Deletion of either/both of the regulatory subunits has no detectable consequences on normal media, but results in specific sensitivity to high concentrations of Na^+ and Li^+ (Bidwai et al., 1994; Reed et al., 1994). Such strains do not display generalized osmosensitivity, and the Na^+ and Li^+ sensitivity is suppressible by high concentrations of K^+ (Bidwai et al., 1994; Reed et al., 1994). These results are consistent with a role for CKII in regulation/maintenance of Na^+ homeostasis in yeast (Glover, 1998). Consistent with the ability of the regulatory subunits to stimulate CKII activity, deletion of either the *CKA1* or *CKA2* gene also results in a similar salt-sensitive phenotype (Glover, 1998).

Temperature-sensitive mutants of *CKA1* and *CKA2* have been constructed and analyzed in the Glover laboratory. All five temperature-sensitive alleles of *CKA2* display

defects in cell cycle progression in both G_1 and G_2/M (Hanna et al., 1995). Flow cytometric analysis of these mutants reveals that CKII might not be required during DNA replication (S phase). Arrest occurs within a single cell doubling following a shift to nonpermissive temperature, and the arrested population consists of approximately equal numbers arrested in G_1 or G_2/M (Hanna et al., 1995). Surprisingly, *cka1* temperature-sensitive alleles do not display a cell cycle arrest similar to that of *cka2* alleles, but instead exhibit a defect in the establishment/maintenance of cell polarity (Rethinaswamy et al., 1998). These alleles continue to grow for 3 to 4 cell cycles after being transferred to non-permissive temperature and then arrest as large, spherical cells with depolarized actin, a morphology that bears striking resemblance to that of the *orb5* alleles of CKII in *S. pombe* (Rethinaswamy et al., 1998). These results are consistent with a functional specialization of the two catalytic subunits of CKII.

An involvement of CKII in the cell cycle has also been demonstrated in mammalian systems. Microinjection of either CKII antisense oligonucleotides or antibody directed against CKII subunits into human fibroblasts inhibits mitogenic stimulation and cell cycle progression through the G_0/G_1, early G_1, and G_1/S transitions (Pepperkok et al., 1994). CKII activity has been reported to be high in G_1 and low in S phase of the cell cycle (Marshak and Russo, 1994). Consistent with a role for CKII in cell cycle progression, CKII is phosphorylated by the cell cycle engine $p34^{cdc2}$ kinase (Litchfield et al., 1995), and several proteins implicated in cell cycle progression have excellent canonical CKII phosphorylation sites (Allende and Allende, 1995).

Overexpression of the catalytic subunits of CKII in *S. cerevisiae* (Padmanabha et al., 1990) or *S. pombe* (Roussou and Draetta, 1994) produces no obvious phenotypic

effects. However, overexpression of the fission yeast regulatory subunit (in *S. pombe*) results in severe growth defects and the formation of multiseptated cells, which implies a defect in cytokinesis (Roussou and Draetta, 1994).

Expression of the catalytic subunit of CKII by itself in lymphocytes of transgenic mice results in stochastic lymphomas, and acute lymphocytic leukemia when coexpressed with the oncogene, c-myc (Seldin and Leder, 1995). Lymphocytes obtained from mice harboring the CKII transgene proliferate abnormally in culture as well. CKII overexpression has been shown to cooperate with p53 deficiency in the development of lymphomas (Landesman-Bollag et al., 1998). Recently, it has also been shown that UV and other stress inducing agents like anisomycin/arsenite/TNF-α achieve activation of p53 by direct association of CKII with p53 and the p38 MAP kinase or the chromatin transcriptional elongation factor, FACT, respectively (Keller et al., 2001; Sayed et al., 2000).

The CKII holoenzyme has been found to interact with a few ribosomal proteins, as well as proteins involved in signalling, proliferation, and development (Guerra and Issinger, 1999). Interestingly, CKIIβ interacts with several proteins in addition to the catalytic subunit of CKII, and several of these proteins, such as $p21^{Waf1/Cip1}$, Mos, A-Raf kinase, and $p90^{rsk}$, are also involved in signal transduction pathways that regulate growth/prolieration (Guerra and Issinger, 1999).

CKII has been predicted to phosphorylate more than 160 proteins, and several of these proteins are involved in DNA replication/repair, transcription, translation, signal transduction, protein folding/synthesis/degradation, apoptosis, ion homeostasis, and cell cycle regulation. One might therefore propose that the physiological role of CKII might

represent regulation of all the cellular processes mentioned above. However, it remains unknown if CKII really phosphorylates all these proteins, and what the role of phosphorylation by CKII might be on these proteins. Critical to an understanding of the true physiological role of CKII would be the identification of regulator/s of the enzyme. Several CKII interacting proteins have recently been reported (Guerra and Issinger, 1999), and testing their individual effect/s on CKII activity/localization might provide insights into the regulatory processes affecting CKII.

In an effort to identify such regulators of CKII, several genetic screens have been undertaken in the Glover laboratory. A multicopy suppressor screen in yeast with *cka2-13* mutants isolated *CDC37* and *ZDS1/2* as genetic interactors of CKII. The purpose of this dissertation is to unravel the molecular mechanism(s) underlying the genetic interactions between CKII, *CDC37*, and *ZDS1/2*. Since Cdc37 is the p50 subunit of Hsp90, and Cdc37 and Hsp90 often cooperate in vivo (Stepanova et al., 1996), my investigation of the CKII/Cdc37 relationship also involved an elucidation of the role played by Hsp90 in the process. A brief review on the current state of knowledge of Hsp90, Cdc37 and Zds1/2 is presented below.

Hsp90

Hsp90 is a member of the subfamily of heat shock proteins that are constitutively expressed in the cytosol but induced to higher levels upon stressful conditions (for reviews see Hendrick and Hartl, 1993; Richter and Buchner, 2001; Young et al., 2001). This 90 KDa molecular chaperone is expressed ubiquitously in all eukaryotic organisms and is highly conserved from bacteria to mammals. Hsp90 has been classified as a

molecular chaperone because it has been shown to promote refolding of denatured proteins in vitro (Yonehara et al., 1996), hold denatured proteins in a folding competent state for other chaperones (Kimura et al., 1997; Yonehara et al., 1996), and prevent unfolding and aggregation (Miyata and Yahara, 1992; Yonehara et al., 1996). In vivo studies have revealed that Hsp90 functions as a molecular chaperone for specific substrate proteins involved in signal transduction events (Richter and Buchner, 2001; Young et al., 2001).

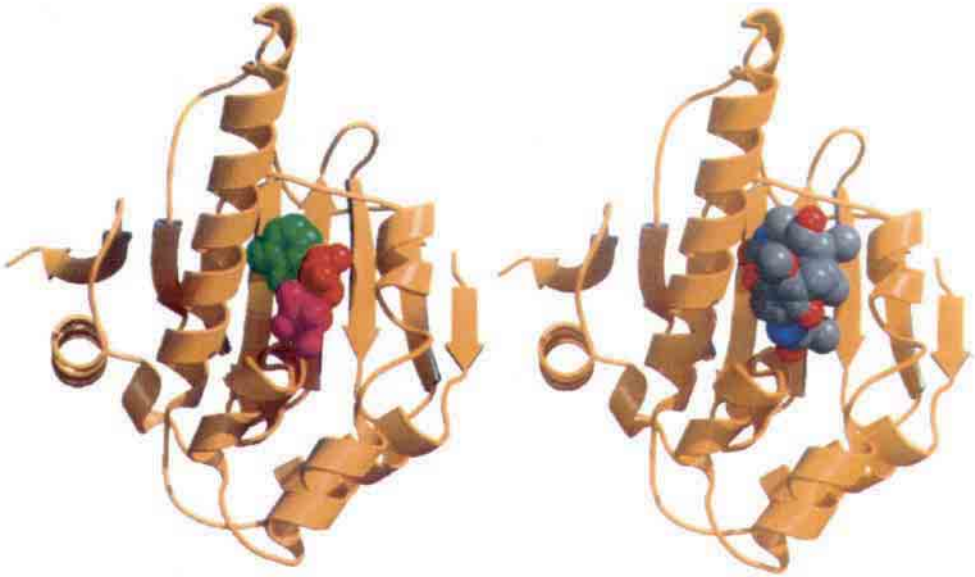
The interaction of Hsp90 with steroid receptors has been well studied. A variety of in vitro and in vivo studies have shown that steroid receptors are complexed with Hsp90. Upon ligand binding, the hormone binding domain (HBD) undergoes a conformational change that results in the release of Hsp90 and the concomitant activation of the steroid receptor (Kimmins and MacRae, 2000 and references therein). In addition to steroid receptor/transcription factors, Hsp90 also chaperones diverse protein kinases thereby affecting the signal transduction pathways of which they are a part (Richter and Buchner, 2001; Young et al., 2001).

Two genes encode closely related isoforms of Hsp90 in mammals as well as in the budding yeast *Saccharomyces cerevisiae*. Deletion of both isoforms in yeast is lethal, indicating that Hsp90 function is essential even at permissive temperatures (Borkovich et al., 1989). Similarly, many mutant alleles of the single *Drosophila HSP90* homolog, *HSP83*, are embryonic lethals over a deficiency of the locus (Van der Straten et al., 1997). In contrast, the *Eschericia coli* homolog of Hsp90, HtpG, appears to be dispensable (Bardwell and Craig, 1988).

The protein folding reaction mediated by Hsp90 is an ATP-dependent process, and the ATPase activity of Hsp90 is essential for its function in vivo (Panaretou et al., 1998). The benzoquinone ansamycin, geldanamycin (GA), has been shown to inhibit Hsp90-dependent ATPase activity in vivo by binding specifically and directly to the nucleotide binding site of Hsp90 (see Fig. 1.3) and disruption Hsp90/substrate complexes (Panaretou et al., 1998; Prodromou et al., 1997; Stebbins et al., 1997). Although originally described as tyrosine kinase inhibitors, benzoquinone ansamycins have been shown to be inactive when added directly to purified tyrosine kinases when added at concentrations >1500 times their effective in vivo dose (June et al., 1990; Whitesell et al., 1992). Additionally, several attempts to demonstrate direct association of ansamycins with tyrosine kinases in vivo and in vitro have been unsuccessful (Miller et al., 1994; Whitesell et al., 1994). Thus, the underlying basis for the inhibitory effects of GA on protein kinases/steroid receptors (only those that are Hsp90 substrates) is believed to be its ability to inhibit Hsp90 ATPase activity.

Hsp90 binds distinct accessory proteins that direct its chaperoning functions toward distinct classes of its substrates. Four distinct Hsp90 containing subcomplexes have been described so far (Caplan, 1999). The Hsp90/Hop/Hsp70/Hip ("Hop complex") and the Hsp90/p23/immunophilin complex ("p23 complex") are associated with the maturation of steroid hormone receptors in successive stages. A third Hsp90 subcomplex comprised of Hsp90/Hsp70/Cns1/Cpr7 has also been described, but its target substrates are not clearly defined. Cdc37 forms a complex with Hsp90 in the absence of any other co-chaperones (Caplan, 1999; Pratt and Toft, 1997), and this complex has been found to

Figure 1.3. Ribbon diagrams of crystal structures of yeast Hsp90 N-terminal domain complexed with Mg^{2+}-ADP on the left and Geldanamycin (GA) on the right. Both the ligands are shown as space-filled models. This figure is taken from Panaretou et al., 1998 (Panaretou et al., 1998).



exist in association with functionally diverse protein kinases. Several of the Hsp90 cochaperones mentioned above bind Hsp90 in the C-terminal region through tetratricopeptide repeat (TPR) sequences (Caplan, 1999; Pratt and Toft, 1997). Cdc37 also binds Hsp90 in the C-terminal region at a site that is adjacent to the TPR acceptor site. In addition to competition between the different TPR-containing proteins for association with Hsp90, the nucleotide binding state of Hsp90 also influences its association with different proteins/co-chaperones (Caplan, 1999; Pratt and Toft, 1997).

Cdc37

Cdc37 was originally identified in *Saccharomyces cerevisiae* as a cell cycle mutant that gives a G_1 cell cycle arrest phenotype (Reed, 1980). Homologs of yeast Cdc37 have been cloned in several organisms including *Drosophila* (21% identity), mammals (21% identity), and humans (20% identity) with the strongest sequence identity confined to the first 30 amino acids. Inspite of the modest homology, *Drosophila* Cdc37 will complement a mutation in the yeast gene (Cutforth and Rubin, 1994).

CDC37 genetically interacts with a remarkable diversity of genes encoding the catalytic subunits of protein kinases involved in diverse functions. In addition to CKII, yeast genetic interactors of *CDC37* include *MPS1* (Schutz et al., 1997), mammalian v-Src when overexpressed in yeast (Dey et al., 1996), *KIN28* (Valay et al., 1995), *STE11* (Abbas-Terki et al., 2000), *CDC28* (Gerber et al., 1995), and *CAK1* (Farrell and Morgan, 2000). *DmCDC37* interacts genetically with the receptor tyrosine kinase encoded by the *sevenless* locus as well as with *Dmcdc2* (Cutforth and Rubin, 1994). In all these cases, overexpression of *CDC37* suppresses a temperature-sensitive mutant of the kinase, or a

reduction in Cdc37 function synthetically enhances the phenotype of a kinase mutant. Mammalian Cdc37 interacts with the cyclin-dependent kinase family of kinases, (specifically CDK4 and CDK6) (Stepanova et al., 1996), Raf1 (Grammatikakis et al., 1999), and Hck, a member of the Src family of kinases (Scholz et al., 2000).

To explain the fact that several genetic screens involving protein kinases yielded *CDC37* as a multicopy suppressor, Cdc37 was initially proposed to be a kinase-specific activator. Indeed, *CDC37* was found to encode the long known, but previously unidentified subunit of an Hsp90 molecular chaperone complex specific for protein kinases (Stepanova et al., 1996). Soon after, yeast Cdc37 was shown to have Hsp90-independent chaperone activity against protein kinase CKII, v-src, and other test substrates in vitro and in vivo (Kimura et al., 1997). Thus, Cdc37 can either cooperate with Hsp90 in mammals or act independently of it in yeast to chaperone protein kinases.

The N-terminal half of mammalian Cdc37 is sufficient for interaction with the catalytic domain of Raf1 (and probably other kinases as well), while the C-terminal region is required for the interaction between mammalian Cdc37 and Hsp90 (at a site that is adjacent to the tetratricopeptide repeat binding site on Hsp90 (Grammatikakis et al., 1999; Silverstein et al., 1998). Thus, the ability of Cdc37 to interact with protein kinases probably reflects its ability to recognize some aspect of the protein kinase fold. However, its interactions with protein kinases are selective in that, for instance, among the cyclin-dependent kinases (CDKs), it interacts with CDK4 and the closely related CDK6 but not with CDK2 (Stepanova et al., 1996).

cdc37-1 mutants and *cdc37-34* mutants in yeast arrest in G1 and G2/M phases of the cell cycle (Dey et al., 1996; Schutz et al., 1997), at least in part because of the failure

of the yeast cell cycle engine, Cdc28, to form productive complexes with the cyclins that activate it (Farrell and Morgan, 2000; Gerber et al., 1995). In addition to Cdc28, Cdc37 function also promotes the stabilization/activation of Cak1 and Mps1, both of which are essential for cell cycle progression in yeast (Farrell and Morgan, 2000; Schutz et al., 1997).

Although, Cdc37 can interact with a variety of protein kinases, we believe that the CKII/Cdc37 connection is unique because of the following observations. The highly conserved amino-terminus of Cdc37 contains an evolutionarily conserved CKII consensus site. This site is phosphorylated by CKII both in vitro and in vivo (Hanna et al., 1995; Kimura et al., 1997), thus raising the possibility that CKII might regulate Cdc37 function.

Cdc37 overexpression has been shown to induce tumors in mice (Stepanova et al., 2000; Stepanova et al., 2000). Cdc37 seems to cooperate with c-myc and cyclin D1 in producing tumors, and the biochemical mechanisms underlying its action are expected to include multiple kinase substrates of Cdc37 that work in concert with the oncogenes to promote proliferation. Proto-oncogenic kinases that Cdc37 has been shown to interact with are v-Src (Dey et al., 1996), Raf-1 (Grammatikakis et al., 1999), and CDK4 (Stepanova et al., 1996). CKII overexpression also produces tumors in mice (Kelliher et al., 1996; Seldin and Leder, 1995), and the mechanism for this might involve its ability to activate Cdc37, which can then activate a host of other oncogenic kinases. It would be interesting to see if mice overexpressing non-phosphorylatable Cdc37 would remain able to cooperate with c-myc or cyclin D1 in promoting oncogenesis.

ZDS1/2

ZDS1 and its homolog, *ZDS2*, were initially isolated as negative regulators of cell polarity (specifically as inhibitors of Cdc42 function; (Bi and Pringle, 1996)). In addition to genetically interacting with *CKA2*, *ZDS1/2* also display genetic interactions with *CDC28,* which encodes the yeast cell cycle engine (Yu et al., 1996); *CEG1*, which encodes the guanylyl transferase responsible for mRNA capping (Schwer et al., 1998); *CDC20*, whose gene product activates the Anaphase Promotion Complex (APC) to catalyze the late metaphase-specific degradation of Pds1 (Wang and Burke, 1997); *SIN4*, which plays a global role in chromatin structure; as well as several other genes involved in multiple processes (Ma et al., 1996; Mizunuma et al., 1998; Roy and Runge, 2000; Wang and Burke, 1997). The incredibly diverse nature of these genetic interactions was the basis for naming this gene pair as *ZDS1* and *ZDS2* (Zillion Different Screens 1 and 2; (Bi and Pringle, 1996)).

ZDS1/2 share no homologies to known proteins and contain no motifs that might give a clue to the nature of their biochemical function. *ZDS1/2* homologs have recently been identified in *S. pombe* (accession number: CAA22593.1) and *C. elegans* (accession number: CAA86458.1). The potential role of the *ZDS* homologs in these organisms has not yet been characterized.

Deletion of *ZDS1* or *ZDS2* alone produces no overt phenotype, but a *zds1Δ zds2Δ* strain has abnormally elongated/hyperpolarized buds, abnormal septin localization, and a cytokinesis defect (Bi and Pringle, 1996). Zds1 localizes to presumptive bud sites and the bud tips in small- and medium-sized buds. Thus, in spite of the diversity of their interactions, *ZDS1/2*, seem to be primarily involved in some aspect of cell polarity. It has

been suggested that the pleiotropic nature of genetic interactions displayed by *ZDS1/2* might simply be a reflection of the pleiotropic nature of Cdc42 itself (Bi and Pringle, 1996). Indeed, Zds1 and Zds2 display two-hybrid interactions with each other, the known Cdc42 effectors Gic1, Gic2, and Cla4, and with other proteins that are likely to function downstream of these effectors (Drees et al., 2001). Consistent with a role in cell polarity, Zds2 also showed two-hybrid interactions with Rho1 and its downstream effectors, Pkc and Bni1 (Drees et al., 2001). Zds2 also interacts with the septin Cdc11, a sporulation-specific protein, Spr6 (Drees et al., 2001), as well as proteins involved in transcriptional silencing, Sir1, Sir2, Sir3, Sir4, and Orc4 (Roy and Runge, 2000).

Although, Zds1/2 display physical and genetic interactions with several proteins involved in cell polarity/Cdc42 function, a role for Zds1/2 in transcriptional silencing is at apparent odds with its role in cell polarity. It is tempting to speculate that the pleiotropy of *ZDS* interactions is indicative of a crucial interaction with CKII, itself one of the most pleiotropic of enzymes (Glover, 1998).

Research Objectives and Approaches

This thesis describes efforts to understand the relationship between protein kinase CKII, the protein kinase-specific chaperone, Cdc37, and the homologous gene pair, *ZDS1/2*. Initial efforts were directed at characterizing the CKII/Cdc37 connection, and probing the relevance of the CKII phosphorylation sites on Cdc37 function using a combination of biochemical and molecular approaches. These studies revealed a positive feedback regulation of CKII and Cdc37, in which the phosphorylation of Cdc37 by CKII is essential for its ability to chaperone CKII as well as multiple other protein kinases. By

regulating Cdc37 phosphorylation, CKII would therefore be expected to play an important role in the maintenance of multiple cellular functions controlled by these kinases. These results are described in more detail in Chapter 2. Chapter 3 describes the underlying molecular basis for the genetic interaction/s of *ZDS1/2* with CKII and *CDC37*. *ZDS1/2* overexpression was found to augment both CKII and Cdc37 function. The ability of Zds1/2 to positively regulate the multifunctional feedback loop between CKII and Cdc37 might explain the observed pleiotropicity of *ZDS1/2*.

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CHAPTER 2

A POSITIVE FEEDBACK LOOP BETWEEN PROTEIN KINASE CKII AND *CDC37* MAINTAINS THE ACTIVITY OF MULTIPLE PROTEIN KINASES[1]

[1]Sricharan Bandhakavi, Richard O. McCann, Claiborne V. C. Glover. To be submitted.

SUMMARY

Cdc37 is a protein kinase-specific chaperone that either acts by itself (Kimura et al., 1997) or in concert with Hsp90 (Stepanova et al., 1996). Cdc37 is phosphorylated by the evolutionarily conserved, ubiquitously distributed Ser/Thr kinase, CKII; and a non-phosphorylatable mutant of *CDC37* has an elongated morphology and slow growing phenotype (McCann et al., In preparation). In this work, we analyzed the importance of Cdc37 phosphorylation by CKII and its potential role in regulating Cdc37 function *in vivo*. We report the construction and characterization of additional CKII phosphorylation-deficient mutants of *CDC37*. *cdc37-S14A* mutants were more severely affected than *cdc37S17A (cdc37M3)* mutants, supporting the idea that the evolutionarily conserved CKII phosphorylation site at serine-14 is more important in regulating Cdc37 function than serine-17. *cdc37-S14A* arrested with reduced CKII activity, and overexpression plasmids expressing *cdc37-S14AS17A* or *cdc37-S14A* were incapable of protecting *cka2-13* mutants on media containing geldanamycin(GA), an Hsp90-specific inhibitor. Incubation of *cka2-13* cells with GA results in reduced phosphorylation of Fpr3 by CKII. Additionally, CKII activity was found to peak during G1 and G2/M phases of the cell cycle, the same phases during which Cdc37 function is essential. Finally, we present genetic evidence that the positive feedback loop between CKII and Cdc37 also maintains the activity of multiple protein kinases.

INTRODUCTION

Protein Kinase CKII is a ubiquitous serine/threonine/tyrosine protein kinase of unknown function. From most sources, the enzyme is composed of two polypeptide subunits, α (35-44 KDa) and β (24-28 KDa), which combine to form an $\alpha_2\beta_2$ tetramer (for reviews see Allende and Allende, 1995; Glover, 1998; Guerra and Issinger, 1999; Pinna and Meggio, 1997). In *Saccharomyces cerevisisae*, the enzyme consists of two distinct catalytic subunits, α and α' (encoded by the genes *CKA1* and *CKA2*, respectively), and two regulatory subunits, β and β' (encoded by the genes *CKB1* and *CKB2*, respectively). CKII recognizes Ser/Thr (or in exceptional cases Tyr; see Wilson et al., 1997) in an acidic environment (Guerra and Issinger, 1999). CKII phosphorylates a broad spectrum of endogenous substrates involved in transcription, translation, signal transduction, etc. Recently, we have shown that *cka2-13* mutants are suppressed by *CDC37* overexpression and that CKII phosphorylates Cdc37 in vivo (McCann et al., In preparation).

Cdc37 was initially isolated in a genetic screen for mutants defective in progression through Start (Reed, 1980). Cdc37 function is required for the proper association of Cdc28 (yeast CDK1) with G1 and mitotic cyclins, thus demonstrating a role for Cdc37 in G2/M progression as well (Gerber et al., 1995). Additional *cdc37* mutants arrest in both G1 and G2/M when shifted to restrictive temperature (Dey et al., 1996). *CDC37* interacts genetically with several different protein kinases that are involved in diverse cellular roles. In addition to CKII, *CDC37* also interacts with *MPS1* (Schutz et al., 1997), mammalian v-Src when overexpressed in yeast (Dey et al., 1996),

KIN28 (Valay et al., 1995), *STE11* (Abbas-Terki et al., 2000), *CDC28* (Gerber et al., 1995) and *CAK1* (Farrell and Morgan, 2000).

To explain the fact that several genetic screens involving protein kinases yielded *CDC37* as a multicopy suppressor, Cdc37 was initially proposed to be a kinase-specific activator. Indeed, *CDC37* was found to encode the long known, but previously unidentified subunit of the Hsp90 molecular chaperone complex (Stepanova et al., 1996). Substrates of Hsp90 include protein kinases as well as steroid receptors, but Cdc37/Hsp90 complexes almost always interact with protein kinases, with one notable exception (Fliss et al., 1997; Silverstein et al., 1998). The N-terminal half of mammalian Cdc37 is sufficient for interaction with the catalytic domain of Raf1 (and probably other kinases as well), while the C-terminal region is required for the interaction between mammalian Cdc37 and Hsp90 (at a site that is adjacent to the tetratricopeptide repeat binding site on Hsp90) (Grammatikakis et al., 1999; Silverstein et al., 1998). Cdc37 has also been demonstrated to possess chaperone activity on its own (independent of Hsp90) in vitro, including against CKII and other test substrates (Kimura et al., 1997).

The CKII-Cdc37 connection is unique among the known genetic interactions between Cdc37 and protein kinases in that the highly conserved amino-terminus of Cdc37 contains an evolutionarily conserved CKII consensus site that is phosphorylated by CKII in vivo (McCann et al., In preparation). *cdc37-S14AS17A* (a mutant allele of Cdc37 in which both of the CKII phosphorylation sites have been mutated) has an elongated morphology and a slow growth phenotype, suggesting that the CKII phosphorylation sites are important for Cdc37 function (McCann et al., In preparation).

We report here the construction and characterization of additional alleles of *cdc37* and present genetic and biochemical evidence for a positive feedback loop between CKII and Cdc37. Although Hsp90 inhibition reduces CKII activity in vivo, Cdc37 and not Hsp90 is the limiting factor in maintaining CKII function. Also, we present genetic evidence supporting the idea that the phosphorylation of Cdc37 by CKII is essential for its ability to maintain the activity of multiple protein kinases involved in diverse cellular functions. This reveals a previously unsuspected role for CKII in regulating the activity of diverse protein kinases (via Cdc37 phosphorylation).

EXPERIMENTAL PROCEDURES

Strains and growth media: *S. cerevisiae* strains used in this study are listed in Table 1. Yeast strains were grown in rich glucose medium (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or in supplemented minimal medium (SMM) (Ausubel, 1987) at different temperatures as indicated in figure legends. Sporulation was carried out in liquid sporulation medium (1% potassium acetate, 0.1% yeast extract and 0.5% glucose). Geldanamycin (Sigma) was dissolved in DMSO and added directly (at indicated concentrations) to warm medium soon after being taken out of the autoclave. Escherichia coli strain DH5α (Clontech) was grown in Luria broth containing 50 μg/ml ampicillin.

Construction of *cdc37* mutant strains: *CDC37* and the four site-directed mutants, i.e., *cdc37M1 (cdc37S14AS17A), cdc37M2 (cdc37S14A), cdc37M3 (cdc37S17A)*, and *cdc37M4 (cdc37S14ES17E)*, were subcloned into BamH1/Kpn1-digested pRS316 (*URA3* marker) and pRS314 (*TRP1* marker). The mutant plasmids were introduced into a *cdc37* null background as well as an isogenic wild type strain as follows: A diploid yeast strain

containing a deletion in one copy of the *CDC37* gene (replaced by *LEU2*, obtained as a gift from David Morgan; ref. Gerber et al, PNAS 92:4651-4655) was transformed with *CDC37* on pRS316, sporulated, and the tetrads dissected. Cells that were auxotrophic for uracil and leucine (i.e., *cdc37* null and rescued by wild-type *CDC37* on pRS316) were selected for further manipulation. After selection of a haploid strain with the **a** mating type, cells were transformed with *CDC37, cdc37M1, cdc37M2, cdc37M3,* and *cdc37M4* (all on pRS314), and the transformants were spotted out at different dilutions on 5-FOA twice (for selection against the presence of the *URA3* gene) followed by selection for remaining nutritional markers to generate strains that were mutant and wild-type for *CDC37*. All strains were tested for growth at a range of temperatures from 23°C to 38°C.

Morphological examinations: Indicated strains were grown to mid-log phase at 23°C and fixed by addition of formaldehyde to a final concentration of 3.7%. For comparison of the mutants generated with *cdc37-1*, SNY 823, SR672 (S. Reed), and AS138-11a (Mark Winey) were used. Cell morphology was visualized using Nomarski optics.

For generation of growth curves, saturated cultures of the relevant strains were inoculated into fresh YPD at a starting concentration of 3×10^5 cells/ml. Cultures were grown at 23°C with vigorous shaking at 450 rpm. To determine cell number, aliquots were removed at the indicated times, fixed with 3.7% formaledehyde, and counted in a hemacytometer.

Strain manipulations: A complete deletion of *PTP1* was constructed by using the PCR method described elsewhere (Wach et al., 1994). Two primers were used to amplify the kanamycin-resistance gene from pFA-6A (Gift from Dr. Philippsen at Biozentrum, Switzerland) together with flanking sequences derived from the immediately upstream

and downstream regions of *PTP1*. The forward primer used was 5'-CAA GAA AAG CGT TTG TTA GTA GTA GTT TGC GAC AGT GGA GCG TAC GCT GCA GGT CGA C-3' and the reverse primer used was 5'-TAT TGA AAA AAT GCA CAA TTA GGA ACT TTT ATA TAG CTG TAT CGA TGA ATT CGA GCT CG-3' (*PTP1* flanking sequences underlined). Disruptions were verified by PCR.

Transformations and other genetic manipulations were done using standard yeast protocols.

Cell cycle synchronization: Cells were grown overnight at room temperature to an optical density of 0.3-0.6. Cells were arrested in G1 or G2/M phases of the cell cycle using YPD supplemented with 10μg/ml α-factor (4 hours) or 30 μg/ml benomyl (4 hours). For S-phase arrest, cells were incubated in YPD containing 0.1 M hydroxyurea for 5 hours. All drug treatments were done at 25°C. In all cases, five A_{600} units of cells were pelleted by centrifugation, brought to a total volume of 100 μl by addition of sterile deionized water and stored at -80°C for Western analysis.

Western blotting/CKII assays: To assay for steady state Tyr-phosphorylation levels of Fpr3 in *cdc37-M2*, the strain (and its isogenic wild type control with and without CKII overexpression plasmids) was grown in 50 ml cultures at 25°C in raffinose (2%w/v) medium lacking uracil. Early log-phase cultures were shifted to 37°C, for 4.5 hours and galactose was added at the time of the shift to a final concentration of 2% (w/v) . Five A_{600} units of each culture were pelleted by centrifugation, brought to a final volume of 100 μl, and stored at -80°C for western analysis. For effects of Geldanamycin on CKII activity, 50 ml YPD cultures of the indicated strains were grown at 26°C to log-phase. Five A_{600} units were collected and incubated in 20 ml of YPD with or without 100 μM

Geldanamycin for 3 hours and washed cells were pelleted and stored as described above. Samples were processed for Western blotting as described elsewhere (McCann et al., In preparation).

Mouse monoclonal anti-Cdc37 (gift from Avrom Caplan, Mt. Sinai School of Medicine) was used at 1:1500, mouse anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology) was used at 1:1000, and rabbit anti-Fpr3 (gift from Jeremy Thorner, UC Berkeley) was used at 1:1000.

Immunodetection was performed using the Immun-star™ detection kit (Bio-Rad).

RESULTS

Hsp90 inhibition reduces CKII activity

Cdc37 has been shown to act as a protein kinase-specific chaperone either by itself (Kimura et al., 1997) or in concert with Hsp90 (Stepanova et al., 1996). Because *CDC37* overexpression acts as a multicopy suppressor of *cka2-13* mutants (McCann et al., In preparation) and Cdc37 and Hsp90 often share common substrates, we sought to observe the effect/s of Geldanamycin (GA), an Hsp90-specific inhibitor, on *cka2-13* mutants. GA is a member of the benzoquinoid ansamycin family of antibiotics that binds Hsp90 within a conserved pocket that constitutes the nucleotide binding site of Hsp90, and inhibits Hsp90 ATPase activity (Panaretou et al., 1998; Prodromou et al., 1997; Stebbins et al., 1997). GA has been shown to indirectly inhibit a variety of oncogenic tyrosine kinases and other substrates of Hsp90 including mineralocorticoid receptor and glucocorticoid receptor (Bamberger et al., 1997; Pratt and Toft, 1997; Segnitz and Gehring, 1997; Uehara et al., 1988; Whitesell et al., 1994). Refolding of denatured

firefly luciferase by the Hsp90 chaperone complex is also inhibited by treatment with ansamycins (Schneider et al., 1996). GA has been shown to inhibit Hsp90 function in vivo in yeast as well (Morano et al., 1999). In contrast to human cell lines, which are sensitive to GA at nanomolar concentrations, the growth of wild-type yeast is unimpaired even at concentrations of 2mM GA (Morano et al., 1999). We have also found similar results with strains wild-type for CKII activity (data not shown).

As shown in Fig. 2.1A, *cka2-13* mutants are GA-sensitive, and this sensitivity is suppressible by Cdc37 overexpression as well as by overexpression of Hsc82 or Hsp82 (the targets of GA in yeast, see Morano et al., 1999; Panaretou et al., 1998). *hsf1-583* strains are marginally sensitive to GA at this temperature (used as a positive control; Morano et al., 1999), while yeast that are wild-type for CKII are unaffected (Fig. 2.1A). *CDC37* overexpression suppressed the GA-sensitivity of *cka2-13* mutants at temperatures from 21°C to 34°C (lowest and highest temperatures tested; data not shown). The minimum restrictive temperature for *cka2-13* mutants is 35°C, and the fact that they do not grow even at 21°C in the presence of GA, emphasizes the importance of Hsp90 function for the survival of *cka2-13* mutants.

Since CKII is an essential enzyme, we wondered if the reduced survival of *cka2-13* mutants in the presence of GA might be due to diminished CKII activity. To test for such a possibility, we sought to examine the in vivo activity of CKII towards Fpr3 (a known CKII substrate in vivo) under these conditions. CKII phosphorylates Fpr3, a yeast nucleolar immunophilin, on Tyr^{184} (Wilson et al., 1997). This phosphorylation is reversed by the protein tyrosine phosphatase, Ptp1 and the *PTP1* gene was deleted in all strains used to measure CKII activity toward Fpr3. Because of the paucity of tyrosine kinases in

yeast, Tyr^{184}-phosphorylated Fpr3 represents a major band in a Western blot of wild-type yeast extracts probed with anti-P-Tyr antibody. As shown in Fig. 2.1B, incubation of *cka2-13* mutants with GA (3 hours) results in reduced Tyr-phsophorylation of Fpr3. We therefore conclude that upon prolonged incubation of *cka2-13* cells with GA, CKII activity drops below a critical threshold causing these cells to die. Since purified Cdc37 has been shown to protect CKII under inactivating conditions in vitro (Kimura et al., 1997), we expect *CDC37* overexpression to maintain CKII activity in the presence of GA (Fig. 2.1A).

Cdc37 and not Hsp90 is the limiting factor in maintenance of CKII

In order to check if *CDC37* overexpression suppressed the GA-sensitivity of *cka2-13* mutants by enhancing Hsp90 function (since enhancement of Hsp90 function by overexpression suppresses *cka2-13*'s GA-sensitivity; see Fig. 2.1A), we made use of the observation by Morano and Thiele that *hsf1-583* cells are sensitive to GA because of reduced Hsp90 function (Morano et al., 1999; Morano and Thiele, 1999). *CDC37* overexpression has been shown to suppress the temperature-sensitivity of *hsp90* mutants in an allele-specific manner (Kimura et al., 1997), raising the possibility that Cdc37 could potentially enhance Hsp90 function in vivo. However, the ability of *CDC37* overexpression to suppress *hsp90* mutants could also be explained by the fact that Cdc37 and Hsp90 share common substrates (and overexpression of Cdc37 might simply protect a few critical common substrates from denaturation in an *hsp90* mutant background). If *CDC37* overexpression enhances Hsp90 function, then it might be expected to suppress the GA-sensitivity of an *hsf1-583* mutant. As shown in Fig. 2.2A, overexpression of *CDC37* did not enhance the ability of *hsf1-583* mutants to grow in the presence of GA,

nor did it suppress its GA-sensitivity at higher temperatures. This argues against a positive influence of *CDC37* overexpression on Hsp90 function. Also, overexpression of either isoform of Hsp90 does not rescue the temperature-sensitivity of a *cka2-13* strain (Fig. 2.2B). Taken together these results imply that *CDC37* overexpression does not enhance Hsp90 function in vivo, and that enhancing Hsp90 function does not protect CKII from inactivation. Thus it is likely that, *CDC37* overexpression suppresses the GA-sensitivity of a *cka2-13* strain by rescuing CKII activity without enhancing Hsp90 function.

Cdc37 overexpression could act as a chaperone towards CKII by itself (as has been shown in vitro (Kimura et al., 1997)); or might still do so through Hsp90, even if the activity of the latter is reduced (but not abolished) upon GA-treatment. However, since Hsp90 overexpression does not suppress the temperature sensitivity of a *cka2-13* strain (only *CDC37* overexpression does), we conclude that although Hsp90 function is required for CKII activity (GA treatment reduces CKII activity, and inhibits *cka2-13* mutants), Cdc37 and not Hsp90 is the limiting factor in maintainng CKII function. Similar findings regarding the nature of the Cdc37/Hsp90 relationship with a temperature-sensitive mutant of the Hck kinase have been reported in human cells (Scholz et al., 2000).

CKII phosphorylation-site mutants of *CDC37* are reduced in Cdc37 function.

We have recently shown that Cdc37 is a substrate of CKII in vivo (McCann et al., 2001) and that a non-phosphorylatable mutant of *CDC37* has an elongated morphology and slow growth phenotype even at 23°C. CKII phosphorylates serine/threonine residues followed by acidic amino acids (Allende and Allende, 1995; Glover, 1998; Guerra and

Issinger, 1999; Pinna and Meggio, 1997). As shown in Fig. 2.3A, the highly conserved amino-terminus of *S. cerevisiae* Cdc37 has two potential CKII phosphorylation sites. Serine-14 is evolutionarily conserved, while serine-17 is not (it is replaced by an acidic amino acid in most higher organisms, making serine-14 an even better consensus CKII phosphorylation site). We have made additional alleles of *CDC37* (Fig. 2.3A) to probe the function of CKII phosphorylation of Cdc37 and also to identify which of the two phosphorylation sites in yeast Cdc37 is more important in regulating Cdc37. *cdc37-S14A* and *cdc37-S17A* test the relative importance of the two sites, while *cdc37-S14ES17E* was made to determine if the presence of a negative charge at the phosphorylation sites is sufficient to mimic a phosphate group.

As previously reported (McCann et al., In preparation), *cdc37-S14AS17A* has a slow-growth phenotype and elongated bud morphology when compared to cells that are wild-type for *CDC37* (Fig. 2.3B and 2.3C). Based on growth curve data, *cdc37M2-S14A* cells take about 3 hours for each doubling at 23°C, while *cdc37-S17A* cells take 2 hours for each doubling at 23°C (doubling time for *CDC37* strain was approximately an hour and 20 minutes; see Fig. 2.3B). Also, the bud morphology of *cdc37-S14A* is more severely affected than that of *cdc37-S17A*. The *cdc37-S14A* strain was found to be temperature-sensitive for growth at 37°C while *cdc37-S17A* was not sensitive for growth at any of the temperatures tested. Taken together, these results support the idea that the evolutionarily conserved CKII phosphorylation site at serine-14 is more important in regulating Cdc37 function than serine-17.

When compared with *cdc37-S14AS17A, cdc37-S14E S17E* displayed a faster growth rate, higher restrictive temperature, and less severe morphological defects (Fig.

2.3B and 2.3C). *cdc37-S14AS17A* takes almost 10 hours to double at 23°C, and *cdc37-S14ES17E* takes approximately 5 hours to double under the same conditions (Fig. 2.3B). Furthermore, the minimum restrictive temperature of *cdc37M1* is 30°C, while *cdc37M4* arrests at 35°C (data not shown). The inability of *cdc37-S14ES17E* to provide wild-type function might reflect the difference between a phospho-serine versus a glutamate residue and/or the necessity of the phosphoryl group to turn over during some stage of the cell cycle.

All temperature-sensitive strains arrested within one cell cycle (data not shown). None of the mutants was reduced in Cdc37 protein levels (Bandhakavi S., unpublished results), supporting the idea that the lack of CKII phosphorylation affects Cdc37 qualitatively rather than quantitatively. This is in contrast to our recent findings (8) that show reduced cdc37M1 levels in a *cdc37-1* background. This discrepancy may reflect the difference in the strain backgrounds used in the two cases.

To characterize the CKII phosphorylation-site mutants of *CDC37* further, the bud morphology of these mutants was compared with that of a previously characterised hypomorphic allele, *cdc37-1* (which encodes a C-terminal truncation of approximately one-third of the protein, Gerber et al., 1995). As shown in Fig. 2.4, the bud morphology of *cdc37-1* (SNY823) is essentially normal at 23°C, but becomes elongated upon prolonged incubation at restrictive temperature (37°C). Similar results were obtained with *cdc37-1* strains in two other backgrounds (SNY823 and AS138-11a). *cdc37M1* displays a dramatic elongated morphology even at 23°C, and this morphology is essentially the same when the strain is shifted to its non-permissive temperature for prolonged periods (see Fig. 2.4). All the *cdc37* mutants described here as well as the

cdc37-1 allele are recessive to wild-type, suggesting that they are loss of function alleles (data not shown). Because of the more extreme elongated phenotype (characteristic of loss of Cdc37 function) displayed by the CKII site mutants of *cdc37,* we conclude that these mutants are more hypomorphic than *cdc37-1* and that the CKII phosphorylation sites are critical for Cdc37 function.

***CDC37* and CKII constitute a positive feedback loop**

Since *CDC37* overexpression suppresses the temperature- and GA-sensitivity of a *cka2-13* mutant (Fig. 2.1), we sought to check if CKII activity was reduced in *cdc37-S14A*. Once again, we looked at the phosphorylation state of Fpr3 (Tyr-P) as an indicator of CKII activity in vivo. Tyr^{184}-phosphorylated Fpr3 represents a major band in a Western blot of wild-type yeast extracts (Fig. 2.5, lane 1) probed with anti-P-Tyr antibody. A slight, but reproducible increase in the intensity of this band is observed upon overexpression of DmCKII in the same strain, and the band is not seen if strains do not carry a *PTP1* deletion, even upon overexpression of DmCKII (Fig. 2.5, lanes 3 and 6). DmCKII expression in yeast has been previously shown to enhance CKII activity towards Fpr3 previously (Wilson et al., 1997). The lack of a large increase in phosphorylated Fpr3 upon DmCKII overexpression likely reflects the occurence of relatively saturated phosphorylation of Fpr3 in wild-type yeast and/or the low turnover rate of phosphorylated Fpr3 (at least under the assay conditions used here).

Extracts made from *cdc37-S14A* arrested at 37°C had a lower level of phosphorylated Fpr3, even upon overexpression of DmCKII (Fig. 2.5, lanes 5 and 6) compared with a strain that is wild-type for *CDC37* function (Fig. 2.5, lanes 1 and 2). Under the assay conditions used here, pools of Fpr3 that were phosphorylated prior to the

shift of *cdc37-S14A* to restrictive temperature might remain phosphorylated even after the shift (since *PTP1* has been deleted). In spite of such a possibility, the intensity of the Tyr-phosphorylated Fpr3 (even in the presence of overexpressed DmCKII) band is clearly reduced in *cdc37-S14A* compared to strains that are wild-type for *CDC37*. Thus, CKII activity is at least reduced (if not abolished) in *cdc37-S14A* upon a shift to restrictive temperature. We conclude that phosphorylation of Cdc37 by CKII at the evolutionarily conserved Ser-14 residue is important for the ability of Cdc37 to protect CKII activity. Consistent with the idea that phosphorylation of Ser-14 is important for the ability of Cdc37 to help maintain CKII activity, overexpression of *cdc37M1(S14AS17A)* or *cdc37M2(S14A)* (unlike wild-type *CDC37* overexpression) was unable to suppress the GA-sensitivity of a *cka2-13* strain (conditions which reduce CKII activity as shown in Fig. 2.1B) as shown in Fig. 2.5B. Overexpression of mutants *cdc37M1 (S14AS17A)*, *cdc37M2(S14A),* or *cdc37M4(S14AS17A)* was unable to suppress the temperature sensitivity of a *cka2-13* or *cdc37-1* strain as well (McCann R., unpublished observations), further indicating that the reduced Cdc37 function in the mutants cripples them from being able to stabilize CKII activity.

The genetic and biochemical results described here support the idea that CKII and Cdc37 exist in a positive feedback loop wherein CKII activates Cdc37 by phosphorylation, which then can activate/maintain CKII (and potentially other kinases as well). Such positive feedback loops constitute an important part of cell cycle regulation. Because CKII is a part of a feedback loop with Cdc37, and function of the latter has been shown to be essential at G1 and G2/M phases of the cell cycle (Dey et al., 1996; Gerber et al., 1995), CKII activity might also peak during the same stages. In order to monitor

CKII activity during the cell cycle, we arrested a strain that is wild-type for CKII in the G1, S, and G2/M-phases of the cell cycle using α-factor, hydroxyurea, and benomyl respectively. As shown in Fig. 2.6, CKII activity toward Fpr3 is high in G1- and G2/M-arrested cells and low in S phase-arrested cells. A requirement for CKII in the G1 and G2/M phases of the cell cycle has been documented previously (Hanna et al., 1995), and another group has previously reported that CKII activity is most likely inhibited during S phase in human cells (using a substrate other than Fpr3) (Marshak and Russo, 1994). Failure to phosphorylate Cdc37 could explain the G1 and G2/M arrest of the *cka2-13* cells.

The CKII/*CDC37* feedback loop helps maintain the activity of multiple protein kinases

The CKII phosphorylation sites on Cdc37 are important for its ability to protect CKII (Fig. 2.5). However, overexpression of DmCKII holoenzyme does not suppress the phenotype of *cdc3-S14AS17A* or *cdc37-S14A* (Bandhakavi S., unpublished results). This raises the possibility that phosphorylation of Cdc37 by CKII might also affect Cdc37's ability to maintain other kinase substrates of Cdc37 as well and that these additional kinases might also be limiting in the CKII phosphorylation site mutants of *CDC37*. In order to test for such a possibility, we made use of the observation that the GA sensitivity of *cka2-13* mutants is suppressed by *CDC37* but not by *cdc37-S14AS17A* or *cdc37-S14A*. Kin28 (Valay et al., 1995), Mps1 (Schutz et al., 1997), and Cdc28 (Gerber et al., 1995) previously have been shown by others to be Cdc37 clients. Because Hsp90 and Cdc37 often share similar kinase substrates (Kimura et al., 1997), we reasoned that temperature-sensitive mutants of *KIN28*, *MPS1*, and *CDC28* might be sensitive to

GA in a fashion similar to the *cka2-13* strain, which loses CKII activity upon incubation with GA (Fig. 2.1B).

The normal restrictive temperature for *mps1-1*, *kin28-ts3*, and *cdc28-109* is reported to be 30°C, 35°C, and 37°C, respectively. All three mutants were found to be GA-sensitive at temperatures lower than their restrictive temperatures (see Fig. 2.7), while their isogenic wild-type controls were insensitive (data not shown). As shown in Fig. 2.7, the GA sensitivity of *kin28-ts3* and *mps1-1* mutants was suppressed by overexpression of *CDC37* but not by *cdc37-S14AS17A* and *cdc37-S14A*. This is consistent with the idea that the CKII phosphorylation sites on Cdc37 are essential for its ability to protect Kin28 and Mps1 as well (in addition to CKII) and that these kinases are reduced in function/activity in both *cdc37M1* and *cdc37M2*.

The GA sensitivity of *cdc28-109* was not suppressed by *CDC37* overexpression (Fig. 2.7) at a variety of temperatures tested (nor was its temperature sensitivity suppressible by *CDC37* overexpression; data not shown). However, we do not think that this suggests that the CKII phosphorylation-site mutants of *CDC37* have adequate Cdc28 function because *cdc37-S14ES17E* is suppressed partially by both Cdc28 overexpression as well as a deletion of *SWE1*, which inhibits the mitotic form of Cdc28 (Bandhakavi S., unpublished observations). *cdc37-1* mutants have been shown to be limiting for Cdc28 function previously (Farrell and Morgan, 2000; Gerber et al., 1995), and *cdc37-1* mutants are synthetically lethal when combined with the *cdc28-109* mutation (Ferguson et al., 1986). Cdc37 function thus is clearly required for Cdc28. However, unlike in *cdc37* mutants (or in *cka2-13* mutants), Cdc37 function might not be limiting in *cdc28-109* mutants. We believe that since Cdc28 overexpression (as well as a deletion of *SWE1*)

suppresses *cdc37-S14ES17E)* the CKII phosphorylation-deficient mutants described here are reduced for Cdc28 function as well. This would be consistent with a model wherein although Cdc37 function is required for Cdc28 activity, it is not the limiting factor for Cdc28 activity/function.

DISCUSSION

We have presented biochemical and genetic evidence for a positive feedback loop between CKII and Cdc37. According to our model (see Fig. 2.8), CKII phosphorylates and activates Cdc37, which in turn helps maintain the activity of CKII. Not only is CKII activity reduced towards a known in vivo substrate, Fpr3, in a CKII phosphorylation-site mutant of *CDC37*, but also the CKII phosphorylation sites on Cdc37 are essential for its ability to suppress the temperature- and GA-sensitivity of a *cka2-13* strain. Additionally, CKII activity peaks at the same stages of the cell cycle that Cdc37 function has been shown to be essential by several groups. In addition to augmenting the activity of Cdc37 and CKII, the positive feedback loop between these two proteins also maintains the activity of additional kinases including, Mps1 (required for spindle pole duplication as well as the spindle checkpoint; see (Schutz et al., 1997), Kin28 (a CTD kinase of RNA Pol II that mediates Pol II's interaction with capping enzymes during transcription of genes; see (Schroeder et al., 2000; Valay et al., 1995), and Cdc28 (the yeast cell cycle engine; see (Gerber et al., 1995). v-Src, and Ste11 also represent substrates of the positive feedback loop since the *cdc37-34* alleles (encoding a ser-14 to leucine replacement, Fliss et al., 1997) have been shown to be defective in the maturation/activation of Ste11, a MAP kinase involved in α-factor signalling in yeast

(Abbas-Terki et al., 2000) and also in v-src activity (Dey et al., 1996). By regulating Cdc37 phosphorylation, CKII plays an important role in maintaining the activity of multiple cellular kinases involved in diverse functions.

Since CKII, Cdc37, and the CKII-phosphorylation site on Cdc37 are evolutionarily conserved, a similar mechanism that positvely regulates multiple kinases (some of which would be homologs of yeast kinase clients/substrates of the CKII/Cdc37 feedback loop shown in Fig. 2.8) may be in place in higher organisms as well. Such a model also gives us a deeper appreciation of the molecular basis for the pleiotropic nature of CKII, since by phosphorylating and activating Cdc37 (which represents only one of the many CKII substrates identified so far), it can play a role in the diverse signal cascades regulated by its client kinases.

Cdc37 overexpression has been shown to induce tumors in mice (Stepanova et al., 2000; Stepanova et al., 2000). Although Cdc37 seems to cooperate with c-myc and cyclin D1 in producing tumors, the biochemical mechanisms underlying its action are likely to be complex and most likely include multiple kinase substrates of Cdc37 that work in concert to promote proliferation. Proto-oncogenic kinases that Cdc37 has been shown to interact with are v-Src (Dey et al., 1996), Raf-1 (Grammatikakis et al., 1999), and CDK4 (Stepanova et al., 1996). CKII overexpression also produces tumors in mice (Kelliher et al., 1996), and the mechanism for this might involve its ability to activate Cdc37, which can then activate a host of other oncogenic kinases. It would be interesting to see if mice overexpressing non-phosphorylatable Cdc37 would remain able to cooperate with c-myc or cyclin D1 in promoting oncogenesis.

How does phosphorylation of Cdc37 by CKII regulate its function? At least two possibilities exist. Phosphorylation might regulate the interaction of Cdc37 with Hsp90 and/or other cochaperones. Alternatively, it might affect the interaction between Cdc37 and its protein kinase client/other substrates. We have been unable to detect protein-protein interactions between yeast Cdc37 and any other protein kinase using a two-hybrid system. Others have had trouble isolating proteins that interact with yeast Cdc37 as well (Farrell and Morgan, 2000). However, there does exist some evidence for the importance of the highly conserved N-terminal half of Cdc37 (which also contains the conserved CKII-phosphorylation site analogous to serine-14 in yeast) since this has been shown to be the region of interaction with at least one protein kinase, Raf-1, in mammalian cells (Grammatikakis et al., 1999). Based on this observation, we suspect that the CKII phsophorylation-deficient mutants of *CDC37* might be impaired in interactions with target protein kinases. Consistent with such a possibility, overexpression of the N-terminal half of yeast *CDC37* has a dominant negative effect in a *cdc37-S14A* but not a *CDC37* strain (Bandhakavi S., unpublished results).

Since a feedback loop between CKII/Cdc37 regulates several other proteins as well and because CKII activity shows a cell cycle-dependent oscillation, we suspect that there might also exist regulators (activators/inhibitors) of the feedback loop. Such gene/s might also be responsible for the oscillation of CKII activity along the cell cycle.

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TABLE 1

Strain	Genotype	Reference/Source
YSB11	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 CDC37*	This study
YSB12	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M1*	This study
YSB13	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M2*	This study
YSB14	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M3*	This study
YSB15	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4*	This study
YSB16	YSB13 *CEN4/ARS1 URA3 GAL1-Dmα GAl10-Dmβ*	This study
YSB17	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 CDC37*	This study
YSB18	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 Hsp82*	This study
YSB19	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 Hsc82*	This study
YDH6	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 CKA2*	Hanna et al., 1995
YDH13	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13*	Hanna et al., 1995
WX241-3b	MAT **a** *mps1-1 ura3-52 his3Δ200 leu2-3*	Mark Winey*

WX257-2b	MAT **a** *ura3-52 his3Δ200 leu2-3*	Schutz et al., 1997
CMY633	MAT **α** *ura3-52 leu2 his3Δ200 cdc28-109* 2μ *URA3 CDC28*	Zarzov et al., 1997
CMY677	MAT **α** *ura3-52 leu2 his3Δ200 cdc28-109*	Zarzov et al., 1997
GF2092	MAT **α** *kin28-ts3 ura3 trp1 leu2 his3*	Valay et al., 1995
GF1067	MAT **α** *ura3 trp1 leu2 his3*	Valay et al., 1995
NSYA	MAT **a** *trp1 leu2 his3 ura3 hsf::LEU2 CEN6/ARSH4 TRP1 HSF*	Morano et al., 1999
NSYB	MAT **a** *trp1 leu2 his3 ura3 hsf::LEU2 CEN6/ARSH4 TRP1 hsf1-583*	Morano et al., 1999
SR672-1	MAT **a** *cdc37-1 ura3 trp1 leu2*	YGSC**
SNY823	MAT **a** *cdc37-1 ura3 trp1 leu2*	S. Reed†
AS138-11a	MAT **α** *cdc37-1 leu2-3, 112 ade2*	Schutz et al., 1997

* Mark Winey, University of Colorado, Boulder.

**Yeast Genetic Stock Center (University of California, Berkeley)

†S. Reed, Scripps Research Institute, La Jolla.

Figure 2.1: Hsp90 inhibition by GA inhibits CKII function. (A) Empty plasmid (lanes 1 and 5) or multicopy plasmids expressing *CDC37* (lanes 2 and 6), *HSC82* (lanes 3 and 6), or *HSP82* (lanes 4 and 8) were transformed into a *cka2-13* strain and spotted on YPD or YPD supplemented with GA (35 μM) at 23°C. *hsf1-583* (NSY B)and YDH6 (wild-type for *CKA2*) were used as controls (bottom row). All cells were spotted at 5000 cells/spot. (B) Indicated strains lacking *PTP1* were grown in minimal medium at 23°C to mid-log phase, and incubated with YPD or YPD supplemented with 100 μM GA at the same temperature for 3 hours. Immunoblots of cell lysates were probed with anti-P-Tyr antiserum (4G10 at 1:1000 dilution) to assay for levels of phosphorylated Fpr3 as a measure of CKII activity in vivo.

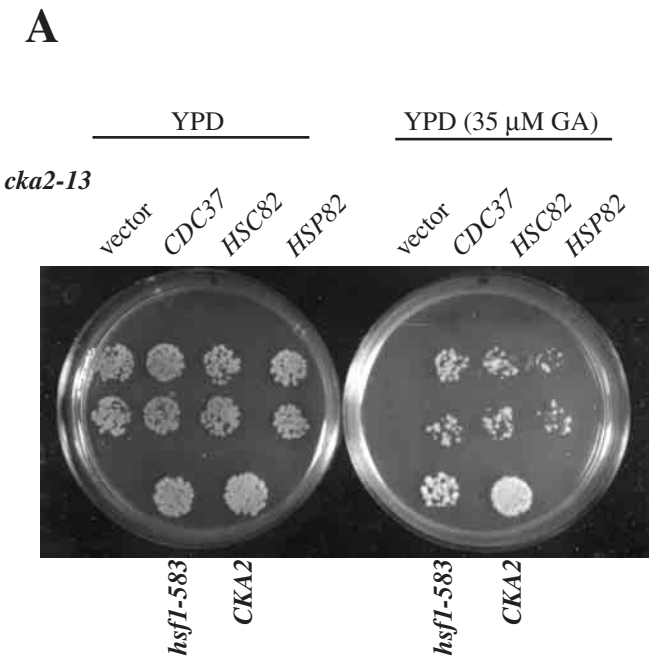

A
YPD
YPD (35 μM GA)
cka2-13
vector
CDC37
HSC82
HSP82
vector
CDC37
HSC82
HSP82
hsf1-583
CKA2
hsf1-583
CKA2
B
CKA2
CKA2 + GA
cka2-13
cka2-13 + GA
Fpr3-P

Figure 2.2: Cdc37 and not Hsp90 is the limiting factor in the maintenance of CKII function. (A) *hsf1-583* cells were transformed with empty plasmid (lanes 1, 2, 5 and 6) or *CDC37* on a multicopy plasmid (lanes 3, 4, 7, and 8) and spotted on YPD without or with GA (35 μM) at 5000 and 500 cells per spot and incubated at 25°C or 30°C for 3 days. (B) *cka2-13* cells transformed with empty vector or multicopy plasmids encoding *CDC37*, *HSP82*, or *HSC82* were streaked out on YPD and assayed for growth at 35°C for 3 days.

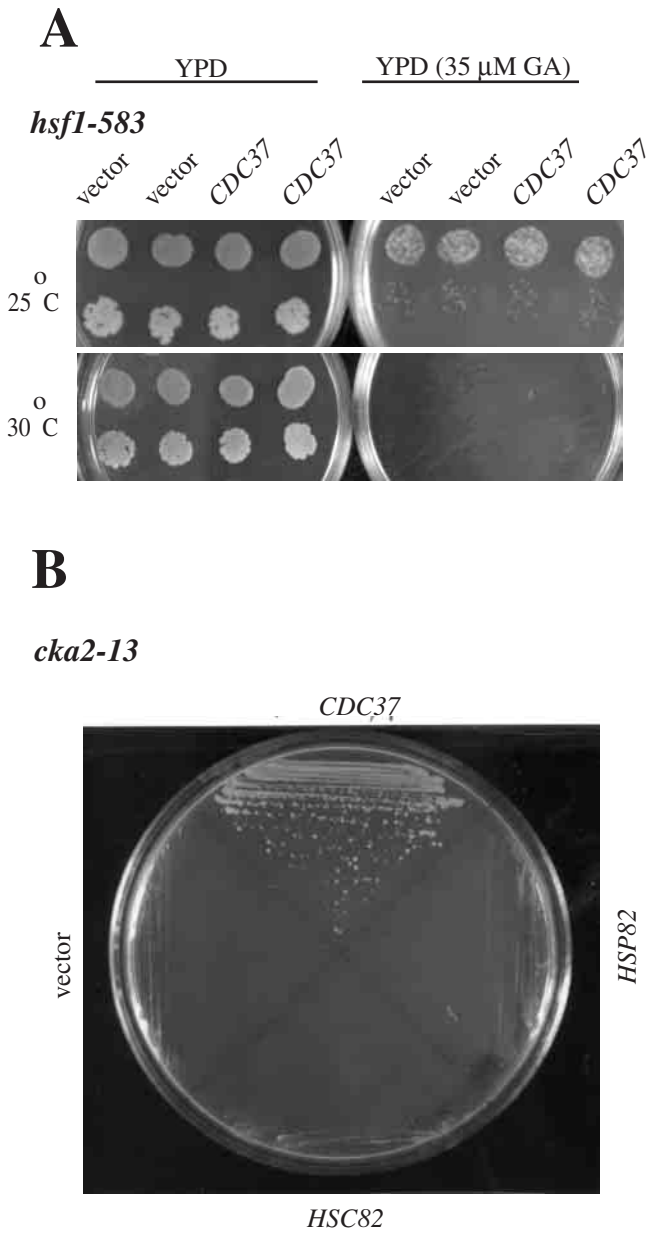
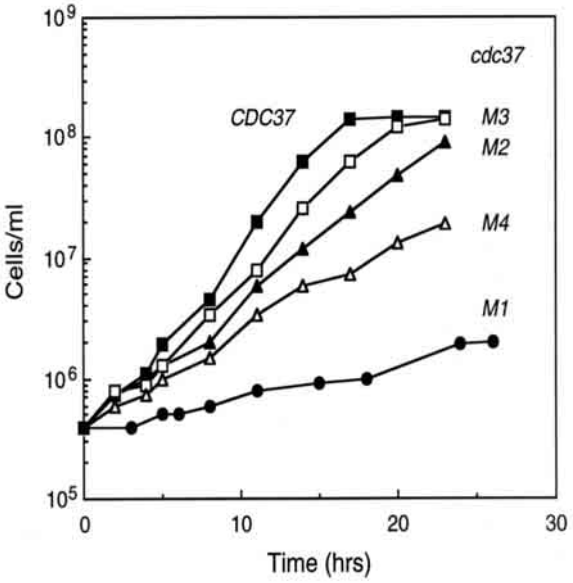

A
YPD
YPD (35 μM GA)
hsf1-583
vector
vector
CDC37
CDC37
vector
vector
CDC37
CDC37
25 °C
30 °C
B
cka2-13
CDC37
vector
HSP82
HSC82

Figure 2.3: CKII phosphorylation-site mutants of *CDC37*. (A) Sequence alignment of the conserved amino-terminus of human, *D. melanogaster* and *S. cerevisiae CDC37* and the site-directed *CDC37* mutations constructed. (B) Growth rates of CKII phosphorylation-site mutants and *CDC37* strains. Indicated strains were inoculated into YPD at a starting concentration of approximately 3×10^5 cells/ml and grown at 23°C. Cell number was monitored as described under "Experimental Procedures." (C) Morphology of CKII phosphorylation-site mutants of *CDC37*. Indicated strains were grown at 23°C to mid-log phase and photographs of cells were taken on a Zeiss IM 35 epifluorescence microscope fitted with Nomarski optics.

A

```
Hs Cdc37   M - V D Y S V W D H I E V S D D E D - E T H P N I D
Dm Cdc37   M - V D Y S K W K N I E I S D D E D - D T H P N I D
Sc Cdc37   M A I D Y S K W D K I E L S D D S D V E V H P N I D

cdc37M1    - - - - - - - - - - - - - - A - - A - - - - - - - - -

cdc37M2    - - - - - - - - - - - - - - A - - S - - - - - - - - -

cdc37M3    - - - - - - - - - - - - - - S - - A - - - - - - - - -

cdc37M4    - - - - - - - - - - - - - - E - - E - - - - - - - - -
```

B



C

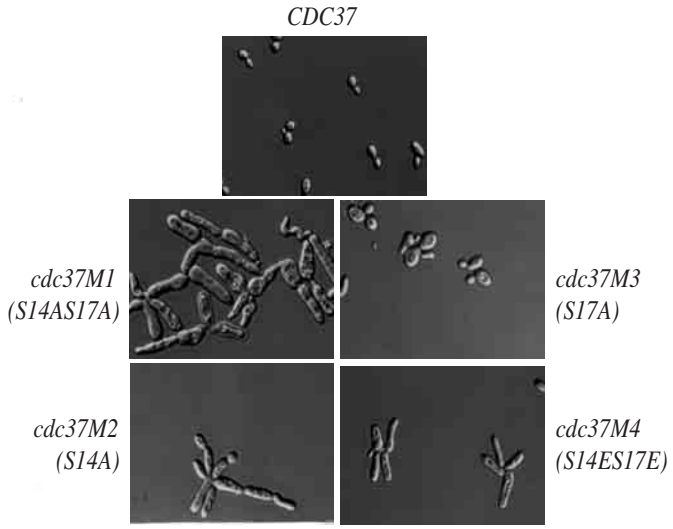


Figure 2.4: Morphology of *cdc37M1(S14AS17A)* and *cdc37-1*. Indicated yeast strains were inoculated into YPD at 23°C at a starting concentration of 3×10^5 cells/ml and grown to mid-log phase at 23°C (lane 1) and fixed in 3.7% formaledehyde or shifted to 37°C (lane 2) at an initial concentration of 10^6 cells/ml for 9 hours, fixed in formaldehyde, and examined by differential interference contrast microscopy.

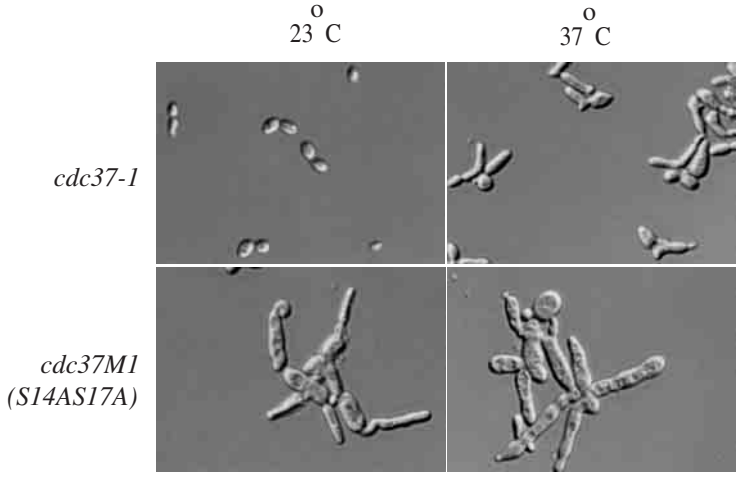

23 °C
37 °C
cdc37-1
cdc37M1
(S14AS17A)

Figure 2.5: CKII phosphorylation-sites on Cdc37 are essential for the ability of Cdc37 to maintain CKII function. (A) Strains *cdc37M2(S14A)* or *CDC37* transformed with an empty plasmid (lanes 1 and 4, respectively) or a plasmid expressing DmCKII (lanes 2, 3, 5, and 6, respectively) were incubated at 37°C for 4.5 hours prior to immunoblotting of cell extracts with anti-P-Tyr antiserum 4G10 (1:1000). Blots were stripped and reprobed with anti-Fpr3 antiserum at 1:1500 dilution. Strains used had the genomic copy of *PTP1* deleted with the exception of the ones used in lanes 3 and 6. (B) *cka2-13* transformed with empty multicopy plasmid (lanes 1 and 5) or multicopy plasmid expressing *CDC37* (lanes 2 and 6), *cdc37M1 (S14AS17A)* (lanes 3 and 7), or *cdc37M2(S14A)* (lanes 4 and 8) were spotted at 5000 cells/spot on YPD or YPD supplemented with GA (35 μM) at 21°C and incubated for 3 days.

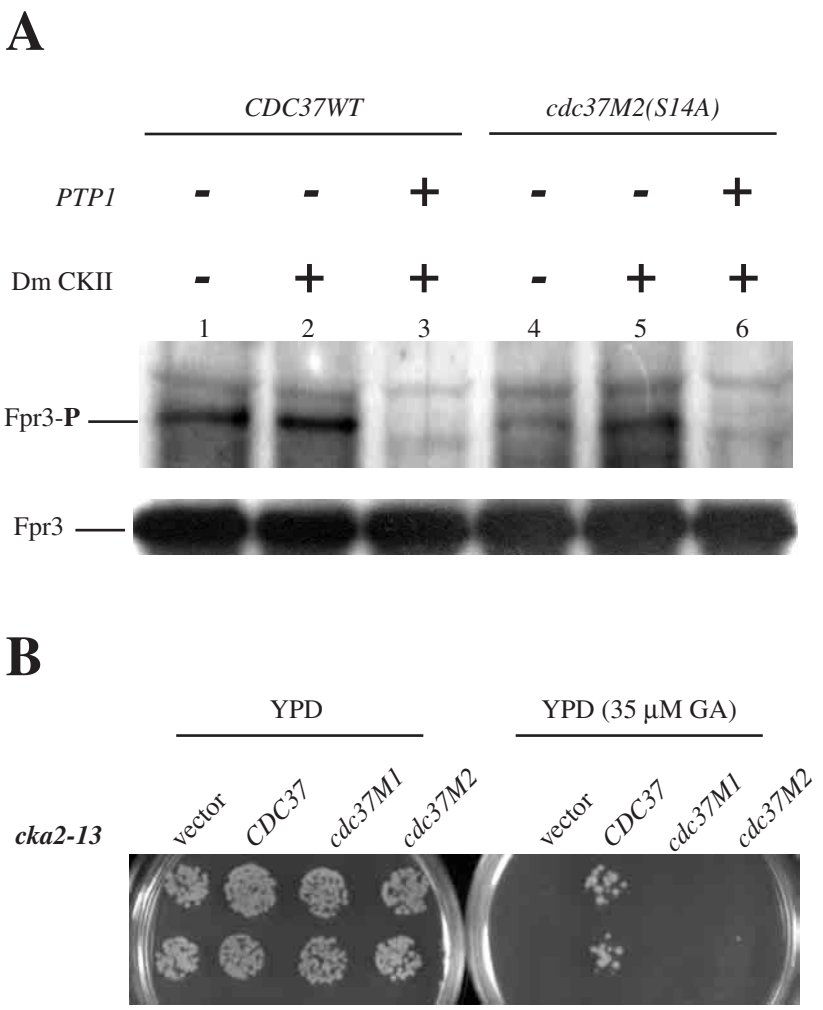

A
CDC37WT
cdc37M2(S14A)
PTP1
- - + - - +
Dm CKII
- + + - + +
1 2 3 4 5 6
Fpr3-P
Fpr3
B
YPD
YPD (35 µM GA)
cka2-13
vector
CDC37
cdc37M1
cdc37M2
vector
CDC37
cdc37M1
cdc37M2

Figure 2.6: Cell cycle stage-specific oscillation of CKII activity towards Fpr3. Extracts were prepared from asynchronous cultures of YDH6 (lane 1) or cultures arrested in G1 (lane 2), S (lane 3), or G2/M (lane 4) as described in "Experimental Procedures," and probed with anti-P-Tyr(4G10) at 1:1000 dilution to assay steady state levels of phosphorylated Fpr3. Blots were stripped and reprobed with anti-Fpr3 (1:1500 dilution). In all cases, the genomic copy of *PTP1* was deleted.

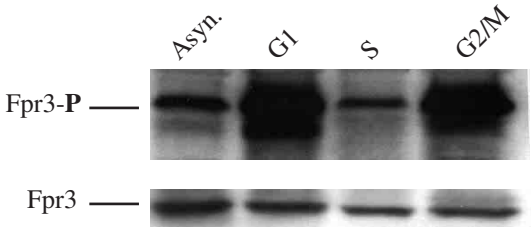

Asyn.
G1
S
G2/M
Fpr3-P
Fpr3

Figure 2.7: CKII phosphorylation sites on Cdc37 are essential for its ability to protect Kin28 and Mps1. Indicated mutant strains were transformed with empty multicopy plasmid (lanes 1 and 5) or multicopy plasmid expressing *CDC37* (lanes 2 and 6) or *cdc37M1(S14AS17A)* (lanes 3 and 7) or *cdc37M2(S14A)* (lanes 4 and 8) and spotted out on YPD or YPD supplemented with GA (35 μM) at the indicated temperature/s for 3 days.

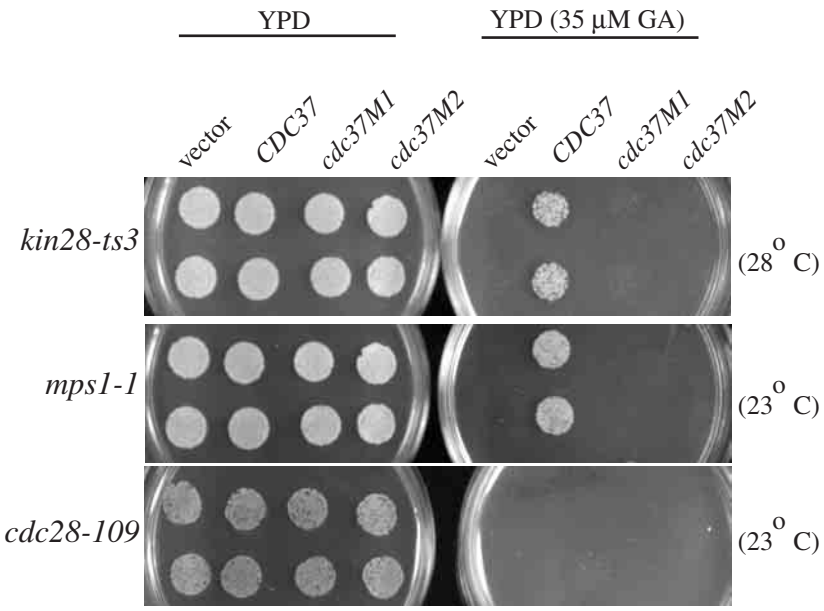
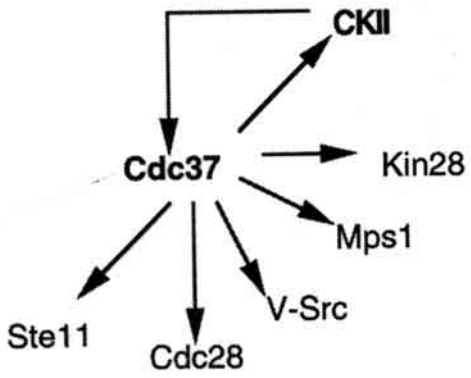

YPD
YPD (35 μM GA)
vector
CDC37
cdc37M1
cdc37M2
vector
CDC37
cdc37M1
cdc37M2
kin28-ts3
(28° C)
mps1-1
(23° C)
cdc28-109
(23° C)

Figure 2.8: A positive feedback loop between CKII and Cdc37 positively regulates multiple protein kinases. A model summarising the interactions between the different genes described in this work.


CKII
Cdc37
Kin28
Mps1
V-Src
Ste11
Cdc28

CHAPTER 3

MOLECULAR BASIS FOR GENETIC INTERACTIONS OF *ZDS1/2* WITH CKII AND *CDC37*[1]

[1]Sricharan Bandhakavi, Richard O. McCann, Claiborne V. C. Glover. To be submitted.

SUMMARY

This report describes our efforts to understand the basis for the observed genetic interaction between protein kinase CKII, and the homologous gene pair, *ZDS1/2,* in *S. cerevisiae*. Phosphorylation of Cdc37 by CKII is essential for its ability to positively regulate CKII as well as other protein kinases. Since *cka2-13* mutants have reduced phosphorylation (and function) of Cdc37, we tested the idea that Zds1/2 might suppress *cka2-13* mutants by enhancing Cdc37/CKII function. Consistent with such a possibility, *ZDS1/2* overexpression was found to suppress the abnormal morphology, temperature- and geldanamycin (GA)-sensitivity of a CKII phosphorylation-deficient mutant of Cdc37, *cdc37-S14A*. Overexpression of *ZDS1/2* also suppresses the GA-sensitivity of a *cdc37-1* allele, showing that the genetic interaction between *CDC37* and *ZDS1/2* is not allele-specific. *ZDS1/2* overexpression was found to increase the amount of Cdc37 protein, which might be the basis for its suppression of *cdc37* mutants. Overexpression of *ZDS1/2* was also found to enhance CKII activity towards Fpr3, a known physiological substrate of CKII in vivo. The ability of Zds1/2 to augment both Cdc37 and CKII function (which together constitute a positive feedback loop) might explain the pleiotropic nature of Zds1/2, since both CKII and Cdc37 regulate diverse cellular functions.

INTRODUCTION

Protein Kinase CKII is a ubiquitous serine/threonine kinase of unknown function. From most sources, the enzyme is composed of two polypeptide subunits, α (35-44 KDa) and β (24-28 KDa), which combine to form an $\alpha_2\beta_2$ tetramer (for reviews, see Allende and Allende, 1995; Glover, 1998; Guerra and Issinger, 1999; Pinna and Meggio, 1997). In *Saccharomyces cerevisisae*, the enzyme consists of two distinct catalytic subunits, α and α' (encoded by *CKA1* and *CKA2*, respectively), and two regulatory subunits, β and β' (encoded by *CKB1* and *CKB2*, respectively). CKII recognizes Ser/Thr (or in exceptional cases Tyr; see Wilson et al., 1997) in an acidic environment (Guerra and Issinger, 1999). CKII phosphorylates a broad spectrum of endogenous substrates involved in transcription, translation, signal transduction, etc.

We have shown that *cka2-13* mutants are suppressed by *CDC37* overexpression and that CKII phosphorylates Cdc37 in vivo (McCann, 1995). *CDC37* encodes the p50 subunit of Hsp90, and acts as a chaperone for protein kinases either by itself or in concert with Hsp90 (Kimura et al., 1997; Stepanova et al., 1996). A positive feedback loop between CKII and Cdc37 positively regulates multiple protein kinases in addition to CKII (Bandhakavi et al., in preparation).

Initially isolated as negative regulators of cell polarity (specifically as inhibitors of Cdc42 function; Bi and Pringle, 1996), Zds1/2 also display genetic interactions with Cdc28 (Yu et al., 1996), *CEG1*, which encodes the guanylyl transferase responsible for mRNA capping (Schwer et al., 1998), *CDC20*, whose gene product activates the Anaphase Promoting Complex (APC) to catalyze the late metaphase-specific degradation of Pds1 (Wang and Burke, 1997), *SIN4*, which plays a global role in chromatin structure,

as well as several other genes involved in multiple processes (Ma et al., 1996; Mizunuma et al., 1998; Roy and Runge, 2000; Wang and Burke, 1997). The incredibly diverse nature of these genetic interactions was the basis for naming this gene pair as *ZDS1* and *ZDS2* (Zillion Different Screens 1 and 2; Bi and Pringle, 1996).

Inspite of the diversity of their interactions, Zds1/2 seem to be primarily involved in some aspect/s of cell polarity. At least three lines of experimental evidence support such an idea. First, a strain bearing a deletion of both *ZDS1* and *ZDS2* has elongated, abnormally shaped buds with hyperpolarized actin (Bi and Pringle, 1996; Schwer et al., 1998; Yu et al., 1996). Second, epitope-tagged Zds1 localizes to presumptive bud sites and the apex of developing buds (Bi and Pringle, 1996). Third, Zds1/2 have recently been reported to physically interact with several gene products involved in cell polarity (Drees et al., 2001). *ZDS1/2* homologs have recently been found in *S. pombe* (accession number CAA22593.1) and *C. elegans* (accession number CAA86458.1).

The basis for the bewildering array of interactions involving Zds1/2 remains ambiguous, however. We have recently reported that a positive feedback loop between CKII and Cdc37 promotes the activity of multiple cellular kinases involved in diverse functions. We now present evidence to show that *ZDS1/2* overexpression augments the function of both CKII and Cdc37, which explains its genetic interactions with these genes. This might also explain the pleiotropic nature of Zds1/2, since both CKII and Cdc37 regulate diverse cellular processes.

EXPERIMENTAL PROCEDURES

Strains and growth media: *S. cerevisiae* strains used in this study are listed in Table 1. Yeast strains were grown in rich glucose medium (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or in YPD medium supplemented with GA at 35μM at different temperatures as indicated in figure legends. Geldanamycin (Sigma) was dissolved in DMSO and added directly (at indicated concentrations) to warm medium. Eschericia coli strain DH5α (Clontech) was grown in Luria broth containing 50 μg/ml ampicillin.

Strain manipulations and cell biology: *PTP1* deletions were carried out as described elsewhere (Bandhakavi et al., in preparation). Transformations and other genetic manipulations where carried out using standard yeast procedures (Ausubel, 1987). For morphological observations, indicated strains were grown to mid-log phase at 23°C and fixed by addition of formaldehyde to a final concentration of 3.7%. Cell morphology was visualized using Nomarski optics.

Flocculation of suppressor and control mutant/wild-type strains was determined as described elsewhere (Padmanabha et al., 1990), except that the cultures were grown in selective liquid media. Use of selective media instead of rich medium minimized variation in flocculation values.

ZDS1 mutagenesis: *ZDS1.1(1-358)* was prepared by introducing a GAA-to-TAA mutation at position 359 using the mutagenic oligonucleotide 5'-CCATGACATGGCCTTAACGATCGTGAC-3'. The AlwN1 selection oligonucleotide (Stratagene) was used as the selection oligonucleotide. Mutagenesis was confirmed by sequencing. Zds1Δ625-809 was produced by removing the 555 nucleotides between the HindIII sites of *ZDS1*, followed by religation.

Western blotting/CKII assays: To assay for steady-state Tyr-phosphorylation levels of Fpr3, the relevant strains were grown at the indicated temperatures to mid-log phase. Five A_{600} units of each culture were pelleted by centrifugation, brought to a final volume of 100 μl, and stored at -80°C for Western analysis. Samples were processed for Western blotting as described elsewhere [McCann, In preparation #109]. Mouse monoclonal anti-Cdc37 (gift from Avrom Caplan, Mt. Sinai Medical Center) was used at 1:1500, mouse anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology) was used at 1:1000, and rabbit anti-Fpr3 (gift from Jeremy Thorner, UC, Berkeley) was used at 1:1000.

Immunodetection was performed using the Amplified Alkaline Phosphatase Immun-star™ kit (Bio-Rad).

RESULTS

A genetic screen was conducted to isolate multicopy suppressors of the temperature-sensitivity of a *cka2-13* allele at 35°C. In addition to *CDC37* (McCann et al., In preparation), we also isolated *ZDS1* as a multicopy suppressor of *cka2* mutants. As shown in Fig. 3.1A (lane 3), overexpression of *ZDS1* suppressed *cka2-13* mutants at 35°C, but not at 37°C. A HindIII deletion construct was fully active as a suppressor as well (Fig. 3.1A, lane 4), indicating that residues 625-809 are dispensable for suppressor function. The introduction of a stop codon at position 359 of the Zds1 protein, which results in the generation of a severely truncated Zds1, was inactive however (Fig. 3.1A, lane 5). Thus residues 359-624 and/or residues 810-915 are essential for the ability of *ZDS1* overexpression to suppress *cka2-13* mutants. Overexpression of *ZDS1* or *CDC37* also suppressed the flocculation (Fig. 3.1B) and slow-growth phenotype of multiple *cka2* alleles (data not shown). Although, *ZDS1* overexpression did not suppress the

flocculation phenotype of *cka2-11* (Fig. 3.1B), it did suppress its temperature-sensitivity and slow growth phenotype (data not shown), indicating that the interaction between *ZDS1* and CKII was not allele-specific.

ZDS1 is not an essential gene, and its homolog, *ZDS2*, was also tested and found to be able to suppress *cka2-13* mutants (data not shown). A strain bearing a deletion of both *ZDS1* and *ZDS2* is viable and has an abnormal bud morphology (Bi and Pringle, 1996; Schwer et al., 1998; Yu et al., 1996). The C-terminal 145 amino acids of Zds1 and the C-terminal 147 amino acids of Zds2 are sufficient for complementation of the abnormal morphology and cold-sensitivity of a *zds1Δ zds2Δ* strain (Schwer et al., 1998). Extrapolating from these results, it seems likely that the residues 810-915 are essential for the ability of *ZDS1* overexpression to suppress *cka2-13* mutants.

We have recently reported a positive feedback loop between protein kinase CKII and the protein kinase-specific chaperone, Cdc37, and that this positively regulates the activity of multiple protein kinases (Bandhakavi et al., in preparation). *cka2-13* mutants arrest with essentially unphosphorylated Cdc37 (Hanna et al., 1995; McCann et al., In preparation), and phosphorylation of Cdc37 by CKII is important for its function in vivo (Bandhakavi et al., in preparation). We initially sought to test if the ability of *ZDS1/2* to interact genetically with *cka2-13* mutants might be reflective of a potential role for these gene/s in the maintenance/regulation of the feedback loop between CKII and Cdc37.

Since *cka2-13* mutants arrest with Cdc37 that is reduced in function, one potential way to suppress *cka2-13* mutants might be to enhance Cdc37 function. Consistent with such a possibility, we found that overexpression of *ZDS1* and *ZDS2*, was able to suppress the temperature sensitivity (Fig. 3.2A, lanes 4 and 5, respectively) and also the abnormal

bud morphology (Fig. 3.2B) of *cdc37M-S14A*. This allele has the evolutionarily conserved, CKII phosphorylation site on serine-14 mutated to an alanine. Unlike the wild-type protein, cdc37-S14A is incapable of protecting multiple kinases (including CKII) under conditions where they lose activity (Bandhakavi et al., in preparation). Although impaired in Cdc37 function, *cdc37-S14A* overexpression suppresses the temperature sensitivity of this strain (Fig. 3.2A, lane 3) better than *ZDS1/2* overexpression does (Fig. 3.2A, lanes 4 and 5). The significance of this result will be discussed later.

As shown in Fig. 3.2B, *ZDS1/2* overexpression also suppresses the abnormal bud morphology of *cdc37-S14E S17E*. This allele has serine-14 and an additional CKII consensus site at serine-17 replaced by glutamate, which partially restores function to a double alanine replacement at these sites (Bandhakavi et al., in preparation). *cdc37-S14A* arrests with reduced CKII activity (Bandhakavi et al., in preparation), and if *ZDS1/2* overexpression simply increases CKII activity in vivo (which could explain why it suppresses *cka2-13* mutants), it could enhance the phosphorylation of serine-17 (which is phosphorylatable in *cdc37M2*) and provide a potential mechanism for *ZDS1/2* to suppress *cdc37* mutants. However, overexpression of *Drosophila* CKII fails to suppress the temperature sensitivity/abnormal bud morphology of *cdc37-S14A* and *cdc37-S14ES17E*(Bandhakavi et al., in preparation), indicating that CKII is not limiting in these mutants, and that the ability of *ZDS1/2* overexpression to suppress these must not involve a simple enhancement of CKII activity alone. Consistent with such an interpretation, *ZDS1/2* overexpression suppresses *cdc37-S14ES17E's* morphology (Fig. 3.2B), and temperature-sensitivity (data not shown).

How might *ZDS1/2* overexpression achieve enhanced Cdc37 function in vivo? To answer this question, we initially used a genetic approach to identify genes which when knocked out would suppress *cdc37* alleles' abnormal bud morphology/temperature sensitivity, and which might be antagonized upon overexpression of *ZDS1/2* in *cdc37* (and also *cka2-13* mutants) to achieve suppression. We found that deletion of *SWE1* (whose product phosphorylates and inhibits the mitotic form of yeast CDK1, see Booher et al., 1993) suppresses the abnormal bud morphology of *cdc37-S14ES17E* (Fig. 3.3). Consistent with other reports, we also found that *ZDS1/2* display an antagonistic relationship with *SWE1* (Ma et al., 1996; Mizunuma et al., 1998; Wang and Burke, 1997); specifically a *SWE1* deletion completely suppresses the abnormal bud morphology of a *zds1Δzds2Δ* mutant (Fig. 3.3). However, a *SWE1* deletion did not suppress any abnormality associated with *cka2-13* mutants (data not shown), arguing against inhibition of *SWE1* by *ZDS1* being the basis for the interaction between *ZDS1* and CKII. Also, deletion of *SWI4* (encoding a component of the SBF (Swi4p-Swi6p) complex that regulates expression at promoters containing the cell cycle box (CCB) elements; Andrews and Herskowitz, 1989) suppressed *cdc37S14A's* abnormal bud morphology, but we observed no genetic interactions between *ZDS1/2* and *SWI4* (data not shown), indicating that *ZDS1/2's* ability to interact with CKII probably did not involve *SWI4* either.

We also sought to test if the genetic interaction between *ZDS1/2* and *CDC37* was allele-specific. In order to test for such a possibility, we made use of our finding that *cdc37* mutants are hypersensitive to geldanamycin (GA), an Hsp90-specific inhibitor (Panaretou et al., 1998; Pratt and Toft, 1997; Prodromou et al., 1997). We expected that

cdc37-S14A might be GA-sensitive because although Cdc37 has been implicated in the maintenance of multiple protein kinases by itself in yeast (Kimura et al., 1997), it often acts in concert with Hsp90 towards certain substrates (Stepanova et al., 1996), and *cdc37-1 hsc82Δ* double mutants exhibit a synthetic growth defect (Kimura et al., 1997). Both *cdc37-1,* which encodes a C-terminal truncation of approximately the last third of the protein (Gerber et al., 1995), and *cdc37-S14A* were found to be hypersensitive to GA (35 μM) (Fig. 3.4A). Overexpression of *ZDS1* and *ZDS2* was able to partially suppress the GA sensitivity of both the *cdc37* mutants tested (Fig. 3.4A), indicating that the genetic interaction between *ZDS1/2* and *CDC37* was not allele-specific.

The ability of *ZDS1/2* overexpression to suppress the GA-sensitivity of *cdc37-1* and *cdc37S14A* raised the possibility that Zds1/2 might enhance Hsp90 function (which in turn might suppress *cdc37* mutants). However, *ZDS1/2* overexpression did not suppress the temperature-sensitivity of *hsp90G170D* or *hsp90T22I* mutants, nor did overexpression of *HSP/C82* (which encode the yeast Hsp90 isoforms) suppress the temperature-sensitivity of *cdc37-1* or *cdc37-S14A* (data not shown). Thus, our results suggest that *ZDS1/2* overexpression enhances Cdc37 function, without enhancing Hsp90 function in vivo.

Western blot analysis of *cdc37-S14A* overexpressing *ZDS1/2* revealed that overexpression (of *ZDS1/2)* caused increased steady state levels of Cdc37 (Fig. 3.4B, lanes 2 and 3), although the increased levels of Cdc37 did not approach the levels attained upon overexpression of *cdc37-S14A* in the same strain background (Fig. 3.4B, lane 4). This coincides well with the enhanced suppression achieved upon overexpression of *cdc37-S14A* in the same strain background (Fig. 3.2A, lane 3) versus

the suppression achieved by overexpression of *ZDS1/2* (Fig. 3.2A, lanes 4 and 5). Thus, we conclude that overexpression of *ZDS1/2* in *cdc37-S14A* achieves suppression by increasing the steady state levels (and hence function) of the partially functional, mutant Cdc37p.

ZDS1/2 overexpression was found to enhance Cdc37 levels in a *cka2-13* strain as well (data not shown). Also, *ZDS1* overexpression (as well as *CDC37* overexpression) was found to suppress the flocculation (Fig. 3.1B) and slow growth phenotype of multiple *cka2* alleles. Because *CDC37* overexpression suppresses the GA-sensitivity of *cka2-13* mutants (Bandhakavi et al., in preparation), we wondered if *ZDS1/2* overexpression might also suppress the GA-sensitivity of *cka2-13* mutants. As shown in Fig. 3.5A, *ZDS1/2* overexpression suppressed GA-sensitivity of a *cka2-13* mutant.

cka2-13 cells have reduced CKII-dependent phosphorylation of Fpr3 upon GA treatment (Bandhakavi et al., in preparation). Thus, in order to suppress *cka2-13* mutants in media containing GA, *ZDS1/2* overexpression might either maintain CKII activity (like *CDC37* has been reported to do; see Kimura et al., 1997) or enhance CKII activity. In order to measure the effect of *ZDS1* overexpression on CKII activity, we looked at the phosphorylation state of Fpr3 (Tyr-P) as an indicator of CKII activity in vivo. CKII specifically catalyzes tyrosine phosphorylation of Fpr3, a nuclear immunophilin, on Tyr^{184}, and this phosphorylation is reversed by the protein tyrosine phosphatase, Ptp1 (Wilson et al., 1997). Hence, measurement of CKII activity towards Fpr3 was carried out in strains carrying a deletion of *PTP1*. Because of the paucity of tyrosine kinases in yeast, Tyr^{184}-phosphorylated Fpr3 represents a major band in a Western blot of wild-type yeast extracts probed with anti-P-Tyr antibody. As shown in Fig. 3.5B (lane 2), the *cka2-*

13 strain arrests at 35°C with very little Tyr-phosphorylated Fpr3. *cka2-13* mutants have a marginally higher activity towards Fpr3 at 33°C (Fig. 3.5B, lane 3), which is the semi-permissive temperature for these mutants. *ZDS1* overexpression (at 33°C) increases the relative amount of Tyr-phosphorylated Fpr3 to a level that is at least comparable to what is seen in the mutant at permissive temperature (Fig. 3.5B, lane 1 versus lane 4). Thus, *ZDS1/2* overexpression can also augment CKII activity.

DISCUSSION

We have presented evidence to support a model in which *ZDS1/2* overexpression results in amplification of both nodes of a recently reported, positive feedback loop between protein kinase CKII and the protein kinase-specific chaperone, Cdc37 (See Fig. 3.6). *ZDS1/2* overexpression suppresses three different phenotypes associated with reduced Cdc37 function in *cdc37-S14A*: temperature-sensitivity, abnormal bud morphology and GA-sensitivity. This interaction is not allele-specific since *ZDS1/2* overexpression also suppresses *cdc37-S14ES17E* and *cdc37-1*. *ZDS1/2* overexpression also suppresses the temperature- and GA-sensitivity of a *cka2-13* mutant and enhances CKII activity in vivo towards a known CKII substrate, Fpr3. *ZDS1/2* overexpression also results in increased protein levels of Cdc37.

Several observations suggest that *ZDS1/2* can activate CKII and Cdc37 independently. The ability of *ZDS1/2* overexpression to augment Cdc37 function must not be due simply to its ability to enhance CKII activity, since overexpression of *Drosophila* CKII by itself does not suppress *cdc37M2* even though it results in increased CKII activity, and *cdc37M2* arrests with reduced CKII activity toward Fpr3 (Bandhakavi

et al., in preparation). *cdc37-S14A* is defective in its ability to maintain multiple protein kinases, and simply increasing CKII activity in these cells would be unable to support/augment all these kinases (since phosphorylation of ser-14 is essential for the ability of Cdc37 to augment multiple kinases; Bandhakavi et al., in preparation). Also, the ability of *ZDS1/2* overexpression to suppress *cdc37-S14ES17E* argues against the potential ability of Zds1 to increase CKII-dependent phosphorylation at Ser-17 as the mechanism behind the suppression of *cdc37* mutants. Additionally, unlike *ZDS1* overexpression, *CDC37* overexpression enhances CKII activity only slightly in a *cka2-13* mutant (data not shown). However, since CKII and Cdc37 also constitute a positive feedback loop, the effects of Zds1/2 on CKII and Cdc37 would also result in a mutual reinforcement of one another. We are currently investigating whether the increased steady-state level of Cdc37 seen in *cdc37-S14A* reflects enhanced expression/ stabilization of Cdc37's half-life.

We were unable to detect a physical interaction between any of the four subunits of yeast CKII or of *CDC37* either with each other or with *ZDS1* using two hybrid analysis (Bandhakavi S., unpublished results). Consistent with the lack of physical interaction between these proteins, the suppression of *cdc37* mutants or *cka2* mutants by *ZDS1/2* is not allele-specific. We hypothesize that Zds1/2 must interact with and potentially modulate the function of some unknown protein that is able to transduce the signal to eventually result in either the activation of CKII and/or enhancement of Cdc37 function. Physical interactors of Zds1 include Zds2 and proteins involved in cell polarity, and Zds2 has been reported to associate physically with a variety of gene products involved in cell polarity and transcriptional silencing (Drees et al., 2001; Roy and Runge, 2000). It

remains to be seen which of these interactions (if any) are necessary for the ability of Zds1/2 to augment CKII/Cdc37 function.

In addition to CKII, the feedback between CKII and Cdc37 positively regulates multiple protein kinases involved in diverse cellular functions (Bandhakavi et al., in preparation). We speculate that this might explain the pleiotropic nature of *ZDS1/2*. CKII phosphorylates and (hence potentially regulates) several proteins (greater than 160 substrates identified so far, see Guerra and Issinger, 1999; Pinna and Meggio, 1997), in addition to Cdc37. By increasing CKII activity in vivo, *ZDS1/2* overexpression can potentially regulate multiple pathways modulated by CKII, in addition to those regulated by Cdc37.

Although *ZDS1/2* enhance CKII activity (and Cdc37 function) in *S. cerevisiae* when overexpressed, it is unknown if they represent the physiological activators of the feedback loop. Arguing against such a role for Zds1/2 are the observations that *ZDS1/2* expression patterns do not show a significant cell-cycle dependent oscillation (Spellman et al., 1998), and that *zds1Δ zds2Δ* mutants are not affected for CKII activity towards Fpr3 or Cdc37 protein levels (Bandhakavi S., unpublished results). However, their overexpression ultimately results in enhanced CKII and Cdc37 function. Therefore, identification of the genes that are essential for *ZDS1/2*'s ability to augment CKII/Cdc37 function in yeast represents an important effort.

Mammalian Cdc37 is expressed in proliferative zones during embryonic development and in adult tissues (Stepanova et al., 2000). Also, human prostatic tumors, neoplasias, and certain pre-malignant lesions display increased Cdc37 expression, suggesting an important role for Cdc37 in prostatic transformation (Stepanova et al.,

2000). Coexpression of Cdc37 with Cyclin D1 or c-*myc* in proliferative zones in mice is tumorigenic (Stepanova et al., 2000; Stepanova et al., 2000). Overexpression of CKII is tumorigenic as well (Kelliher et al., 1996). Since CKII and the CKII phosphorylation site on Cdc37 are conserved evolutionarily, it raises the possibility that the feedback loop between CKII and Cdc37 is also conserved, and that misregulation of the feedback loop could explain the induction of tumors upon overexpression of CKII or Cdc37. It remains to be seen if the regulation of this pathway by *ZDS1/2* might be conserved as well. *ZDS* homologs have recently been reported in *S. pombe* and *C.elegans*, although no homologs in higher organism have been reported yet.

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TABLE 1

Strain	Genotype	Reference
YRM2.2	MAT a *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13 2μ URA3 ZDS1*	This study
YRM 2.3	MAT a *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13 2μ URA3 zds1Δ625-809*	This study
YRM2.4	MAT a *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13 2μ URA3 zds1.1-358*	This study
YSB11	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 CDC37*	This study
YSB13	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M2*	This study
YSB15	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4*	This study
YSB20	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M2 2μ URA3 cdc37M2*	This study
YSB21	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M2 2μ URA3 ZDS1*	This study
YSB22	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M2 2μ URA3 ZDS2*	This study
YSB23	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4 2μ URA3 cdc37M4*	This study
YSB24	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4 2μ URA3 ZDS1*	This study
YSB25	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4 2μ URA3 ZDS2*	This study
YSB26	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 CDC37 SWE1::KanMX4*	This study

YSB27	MAT **a** *leu2 ura3 trp1 CDC37::LEU2 CEN6/ARSH4 TRP1 cdc37M4 SWE1::KanMX4*	This study
DY3143	MAT **a** *zds1::URA3 zds2::TRP1 ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1*	Yu et al.
YSB28	MAT **a** *zds1::URA3 zds2::TRP1 ade2-1 his3-11,15 leu2-3 trp1-1 ura3-1 SWE1::KanMx4*	This study
SR672-1	MAT **a** *cdc37-1 ura3 trp1 leu2*	YGSC*
YSB29	MAT **a** *cdc37-1 ura3 trp1 leu2 2μ URA3 CDC37*	This study
YSB30	MAT **a** *cdc37-1 ura3 trp1 leu2 2μ URA3 ZDS1*	This study
YSB31	MAT **a** *cdc37-1 ura3 trp1 leu2 2μ URA3 ZDS2*	This study
YSB17	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 CDC37*	This study
YSB32	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 ZDS1*	This study
YSB33	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13* 2μ *URA3 ZDS2*	This study
YDH6	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 CKA2*	Hanna et al.
YDH13	MAT **a** *ura3 Δcka1::HIS3 Δcka2::TRP1 CEN6/ARSH4 LEU2 cka2-13*	Hanna et al.

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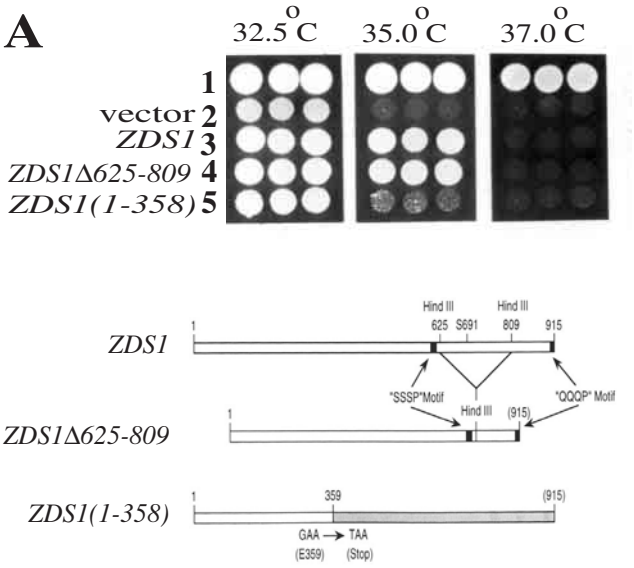
Figure 3.1: Multicopy suppression of *cka2^{ts}* by *ZDS1* and *CDC37*. (A) Analysis of *ZDS1* mutants capable of suppressing *cka2-13*. Strains used in order were, YDH6 transformed with pRS426 (lane 1), YDH13 transformed with pRS426 (lane2), or YDH13 transformed with pRS426 expressing *ZDS1* (lane 3), or *ZDS1(1-358)* (a severely truncated version of *ZDS1*, lane 4), or *ZDS1Δ625-809* (lacking residues 625-809, lane 5). (B) The effect of *ZDS1* and *CDC37* on the flocculation phenotype associated with three *cka2^{ts}* alleles was determined. The indicated strains were transformed with pRS426 (lanes 1), or *ZDS1*/pRS426 (lanes 2), or with *CDC37*/pRS426 (lanes 3).


A
32.5°C
35.0°C
37.0°C
1
vector 2
ZDS1 3
ZDS1Δ625-809 4
ZDS1(1-358) 5
ZDS1
Hind III
Hind III
625
S691
809
915
"SSSP" Motif
"QQQP" Motif
Hind III
(915)
ZDS1Δ625-809
359
(915)
ZDS1(1-358)
GAA → TAA
(E359)
(Stop)

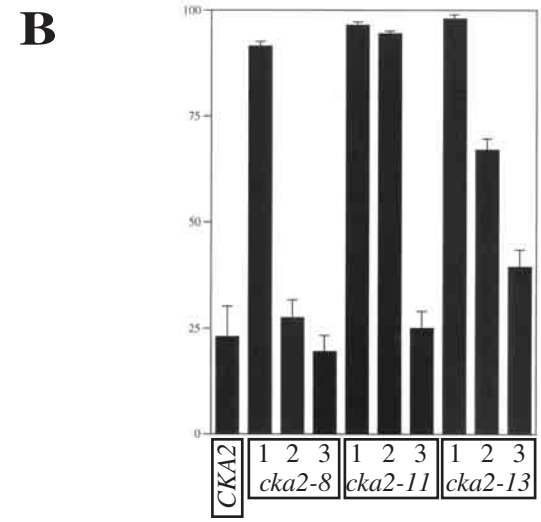

B
100
75
50
25
0
CKA2
1 2 3
cka2-8
1 2 3
cka2-11
1 2 3
cka2-13

Figure 3.2: Multicopy suppression of *cdc37* mutants by *ZDS1/2*. (A) *cdc37M2(S14A)* was transformed with pRS426 (row 2), or pRS426 expressing *cdc37M2* (row 3), or *ZDS1* (row 4) or *ZDS2* (row 5) and spotted at 5000 cells per spot at the indicated temperatures for 3 days before being photographed. An isogenic strain wild-type for *CDC37* was transformed with pRS426 and spotted similarly in row 1. (B) *ZDS1/2* overexpression suppresses abnormal bud morphology associated with *cdc37M2(S14A)* or *cdc37M4(S14E S17E)*. Indicated strains bearing relevant overexpression plasmids as indicated were grown at 27°C to mid-log phase and photographs of cells were taken on a Zeiss IM 35 epifluorescence microscope fitted with Normarski optics.

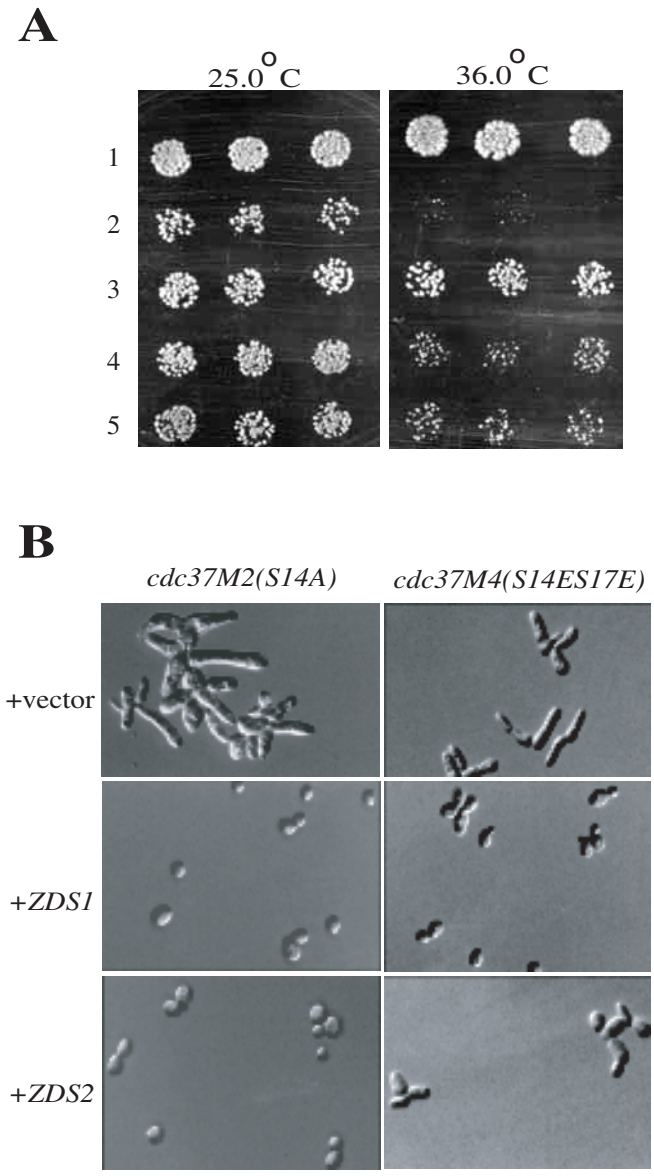

A
25.0°C
36.0°C
1
2
3
4
5
B
cdc37M2(S14A)
cdc37M4(S14ES17E)
+vector
+ZDS1
+ZDS2

Figure 3.3: Abnormal bud morphology of *cdc37M4(S14E S17E)* and *zds1Δ zds2Δ* is *SWE1*-dependent. Indicated strains were grown at 27°C to mid-log phase in minimal medium and photographed on a Zeiss IM 35 epifluorescence microscope fitted with Normarski optics.

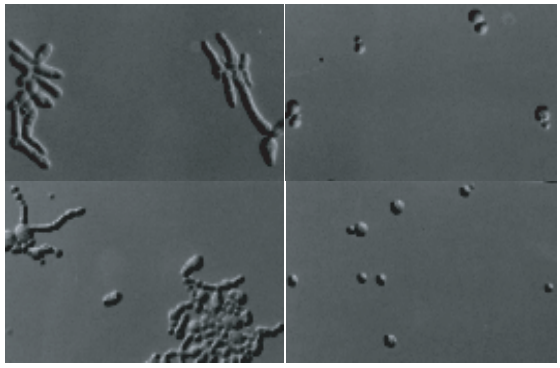
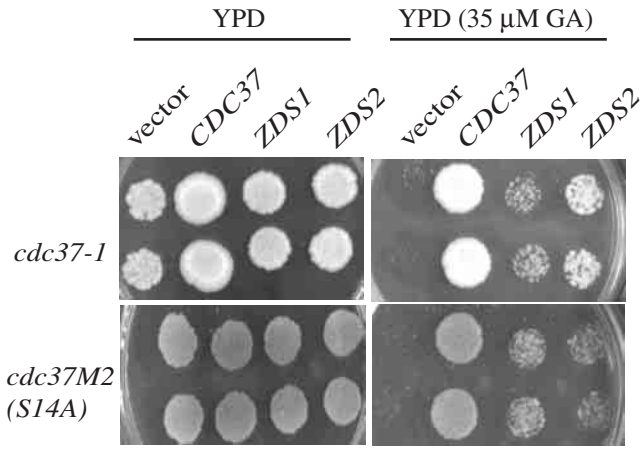

cdc37M4
cdc37M4 swe1Δ
zds1Δ zds2Δ
zds1Δ zds2Δ swe1Δ

Figure 3.4: *ZDS1/2* overexpression enhances Cdc37 function by increasing Cdc37 protein levels. (A) *cdc37-1* (panel 1, at 27°C) and *cdc37M2(S14A)* (panel 2, at 30°C) transformed with pRS426 or pRS426 expressing *CDC37*, *ZDS1* or *ZDS2* were spotted on YPD or YPD supplemented with GA (35 μM) and incubated at the indicated temperatures for 3 days and photographed. (B) *cdc37M2(S14A)* cells carrying empty vector (lane 1), or overexpressing *ZDS1* (lane 2), or *ZDS2* (lane 3), or *cdc37M2(S14A)* (lane 4) were grown to mid-log phase at 27°C, and immunoblots of cell lysates were probed with mouse monoclonal anti-Cdc37 antiserum at 1:1000 dilution .

A


YPD
YPD (35 μM GA)
vector
CDC37
ZDS1
ZDS2
vector
CDC37
ZDS1
ZDS2
cdc37-1
cdc37M2
(S14A)

B

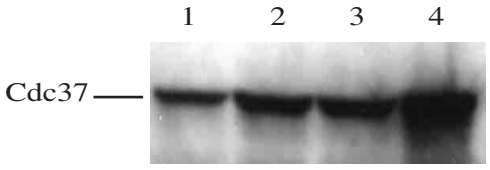

1
2
3
4
Cdc37

Figure 3.5: *ZDS1/2* overexpression suppresses GA-sensitivity of *cka2-13* and enhances CKII activity towards Fpr3 in vivo. (A) *cka2-13* cells transformed with pRS426 or pRS426 expressing *CDC37*, *ZDS1* or *ZDS2* were spotted at 5000 cells/spot on YPD or YPD supplemented with GA (35 μM) and incubated at 21°C or 30°C for 3 days. (B) *cka2-13* strain transformed with pRS426 (26°C, 35°C, and 33°C in lanes 1, 2, and 3, respectively) or pRS426/*ZDS1* (at 33°C, lane 4) was grown to mid-log phase at the temperatures indicated. For cultures incubated at 35°C, half of the culture grown at 26°C was shifted to 35°C for 3 hours.

Cell lysates of the indicated strains were probed with anti-P-Tyr antibody (4G10 at 1:1000 dilution) to assay for levels of phosphorylated Fpr3 as a measure of CKII activity in vivo.

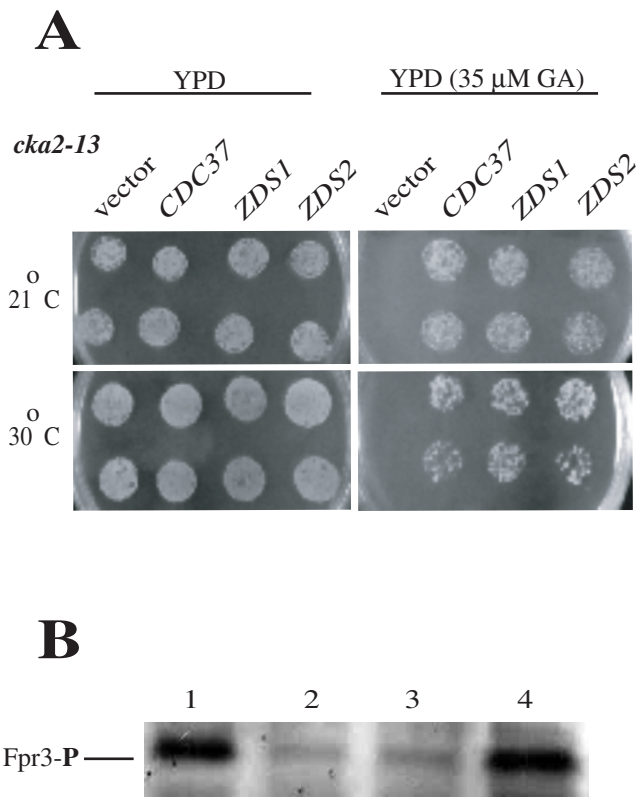
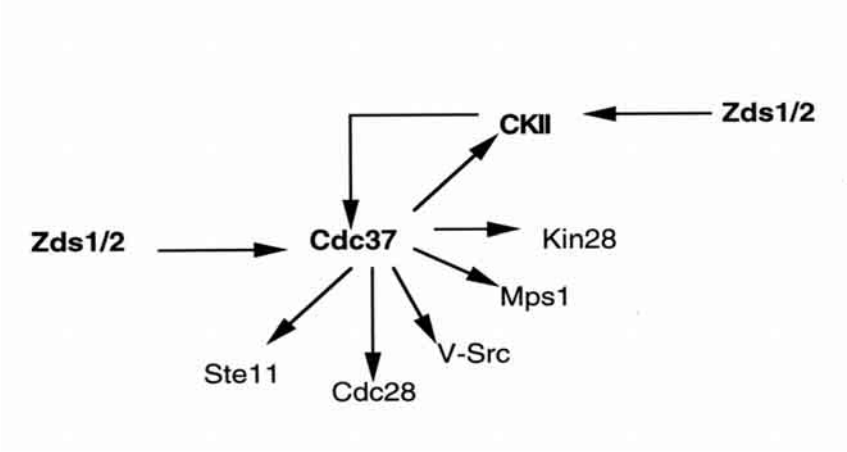

A
YPD
YPD (35 μM GA)
cka2-13
vector
CDC37
ZDS1
ZDS2
vector
CDC37
ZDS1
ZDS2
21 °C
30 °C
B
1
2
3
4
Fpr3-P

Figure 3.6: A model depicting the observed interactions between *ZDS1/2* and the positive feedback loop between CKII and Cdc37.


CKII
Zds1/2
Zds1/2
Cdc37
Kin28
Mps1
V-Src
Ste11
Cdc28

CHAPTER 4

CONCLUSION

The work presented in this dissertation provides a deeper understanding of the molecular basis for the pleiotropic nature of protein kinase CKII, the nature of its relationship with Cdc37, and the underlying basis for the genetic interaction of *ZDS1/2* with CKII and Cdc37.

Biochemical and genetic evidence that supports the existence of a positive feedback loop between CKII and Cdc37 is described in Chapter 2. According to this model, CKII phosphorylates and activates Cdc37, which in turn augments the activity of CKII as well as other protein kinases involved in diverse cellular functions. Therefore, by regulating Cdc37 phosphorylation, CKII would be expected to play an important role in the cellular functions regulated by these kinases.

CKII has been predicted to phosphorylate more than 160 proteins (Cdc37 is just one of these), and many of these proteins are involved in DNA replication/repair, transcription, translation, signal transduction, protein folding/synthesis/degradation, apoptosis, ion homeostasis, and cell cycle regulation (Guerra and Issinger, 1999). While possible, the task of mutagenizing all CKII phosphorylation sites on all of these proteins to probe the relevance of CKII phosphorylation on each of them (or the functions controlled by these proteins) is daunting. Because of this, identification of regulator/s of the enzyme may be critical to an understanding of the true physiological role of CKII. But is the enzyme activity regulated?

Also described in Chapter 2 is a cell cycle stage-specific oscillation of CKII activity towards Fpr3, a known CKII substrate in vivo. CKII activity was found to be highest towards Fpr3 in G1- and G2/M-arrested cells and lowest in S phase-arrested cells. Using a substrate other than Fpr3, Daniel Marshak's group has previously shown that CKII activity towards Cdc2 is higher in G1 than S phase in mammalian cells (Marshak and Russo, 1994). A requirement for CKII in the G1 and G2/M phases and not the S-phase has also been demonstrated by flow cytometric analysis of *cka2-13* mutants generated in the Glover laboratory (Hanna et al., 1995). Thus, it seems likely that the enzyme is regulated in vivo, which implies the existence of physiological activators/inhibitors of CKII.

The proposed existence of regulators of CKII activity is at apparent odds with the "constitutively active" crystal structure of the CKII catalytic subunit/holoenzyme (Niefind et al., 2001; Niefind et al., 1998). However, the reduced phosphorylation of CKII substrates in S-phase could be explained by a mechanism that takes into account the constitutively active structure of the enzyme. CKII localisation patterns/protein levels might be regulated in a cell cycle-specific fashion by specific proteins (that would also serve to inhibit CKII activity), or protein phosphatases might dephosphorylate CKII substrates at a higher rate in S-phase than G1 or G2/M. The second possibility seems unlikely at least in the case of Fpr3, since strains used to assay the state of phosphorylation of Fpr3 had the genomic locus of *PTP1* (the phosphatase that dephosphorylates Fpr3) deleted. A third and more interesting possibility is the possible existence of an inhibitory protein that binds to CKII and blocks its catalytic site from

being available to its substrates. Isolation of CKII-interacting proteins during each of the cell cycle stages might identify the relevant inhibitor of CKII activity.

Interestingly, *ZDS1/2* overexpression was found to amplify both nodes of the positive feedback loop between CKII and Cdc37 (see Chapter 3). These effects are most likely indirect since no physical interactions were detected between any of the four subunits of CKII or Cdc37 with Zds1. However, since the CKII/Cdc37 feedback loop has pleiotropic effects and CKII phosphorylates several proteins in addition to Cdc37, it is interesting to speculate that the pleiotropic nature of Zds1/2 can be explained by its effects on CKII/Cdc37.

Several physical interactors of Zds1/2 have recently been reported (Drees et al., 2001). It would be interesting to see which, if any, of these interactions are required for Zds1/2 to augment CKII/Cdc37 function. CKII, Cdc37, the CKII phosphorylation site/s on Cdc37, and Zds1/2 are conserved evolutionarily. Thus, it is possible that not only is the feedback loop between CKII and Cdc37 conserved in higher organisms, but also its regulation by Zds1/2. Identification of the specific genes responsible for this regulation would provide further insight into the regulation of CKII, as well as the pleiotropic nature of Zds1/2 and CKII.

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