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CKS PROTEINS IN CELL CYCLE REGULATION

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ABSTRACT

Eva Rose M. Balog

Cks Proteins in Cell Cycle Regulation

Eukaryotic cells coordinate the processes of cell growth and division through the activity of cyclin-dependent kinases (Cdks). Cdks generate immense signaling specificity and complexity by modifying hundreds of substrates via reversible phosphorylation. Many Cdk substrates are multiphosphorylated, but the mechanisms underlying the enzymatic activity of Cdks toward such substrates are not fully understood. Cks1 is a small essential protein that physically associates with Cdks, possesses a phosphate-binding pocket, and acts as both a positive and negative regulator of Cdk activity. Cks1 has also been extensively studied for its ability to form domain-swapped homodimers, the formation of which is controlled by two conserved proline residues. This dissertation presents research that combined x-ray crystallography, enzyme kinetics, and biophysical methods to demonstrate a direct role for Cks1 in targeting Cdks to multiphosphorylated substrates and develop a new hypothesis for the conservation of a proline residue that mediates domain-swapping in Cks1.

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Chapter 1: Introduction

1.1. Background

Eukaryotic cell duplication and division occurs via a highly coordinated series of events collectively called the cell cycle. Cells have evolved a control system that ensures that contents are duplicated and separated with spatial and temporal fidelity. Cyclin-dependent kinases (Cdks) are key regulators of the cell cycle (Morgan 1995). Cdks drive the events of the cell cycle by associating with cell cycle stage-specific cyclin subunits and phosphorylating their hundreds of substrates at specific phosphorylation sites, in response to specific cues, to elicit specific effects. The molecular basis of how Cdks manage to execute the events of the cell cycle with such great precision is of great interest to both basic science and cancer research, since deregulation of cell cycle control leads to insensitivity to antigrowth signals, a hallmark of cancer (Hanahan and Weinberg 2000). In a 2000 review entitled "A Long Twentieth Century of the Cell Cycle and Beyond", Sir Paul Nurse wrote, "Given the importance of CDKs in cell cycle control, it is surprising how little is known about the interface between CDK activities and the implementation of cell cycle events." (Nurse 2000). Nurse predicted that in the next 10-20 years, work would focus on molecular mechanisms by which Cdks initiate DNA replication and mitotic events, informed by structural studies of the regulation of Cdk activity, the effects of phosphorylation, and Cdk interaction with substrates and inhibitors. This dissertation is my small contribution to the next century of cell cycle research.

1.2. Organization

In Chapter 1, I will introduce the reader to the questions I have asked and tried to answer and the approach I have employed. Chapter 1 will also introduce the molecular players and briefly summarize the biological contexts in which they act. Chapter 2 describes the expression, purification, and activation of the major yeast mitotic cyclin/Cdk complex, Clb2/Cdk1. Chapter 2 also includes experiments designed to determine the contribution of the Cdk-subunit Cks1 to the kinetics of phosphorylation. Chapter 3 tells the story of a mutant construct of Cks1 whose structure has informed our understanding of Cks1 conservation and function. This chapter is largely based on previously published material (Balog et al. 2011). Chapter 4 explores the specificity of Cks1-phosphoprotein interaction.

Throughout this dissertation fusion proteins will be indicated with a dash ("-") and protein complexes will be indicated using a slash ("/"). Unfortunately convention dictates that orthologous proteins and analogous residues are also denoted with a slash, e.g. "Cks1/suc1" or "Pro93/Pro62". Efforts were made to provide sufficient context for comprehension.

1.3. Objectives

Swe1 (*Saccharomyces* Wee1) is a multiply phosphorylated tyrosine kinase that phosphorylates and inhibits Clb2/Cdk1 prior to mitotic entry in budding yeast. The role of Swe1 in the coordination of cell size and cell division is of great interest in cell cycle research. Specifically, Swe1 undergoes hyperphosphorylation at the G2/M transition, but the functional significance and

signaling outputs of Swe1 phosphorylation, as well as the kinases responsible, are not completely understood. In 2005, the Kellogg laboratory at UCSC published a paper presenting a model of Swe1 phosphoregulation that showed for the first time that Swe1 is itself a substrate of its own substrate, Clb2/Cdk1 (Harvey et al. 2005). Their model proposes that in late G2, Swe1 is partially phosphorylated and activated by Clb2/Cdk1. This partial phosphorylation is required for the formation of a stable complex between Swe1 and Clb2/Cdk1 that maintains Clb2/Cdk1 inactivity. As levels of Clb2 rise throughout late G2, increasing Clb2/Cdk1 activity leads to hyperphosphorylation of Swe1 and dissociation of the Swe1/Clb2/Cdk1 inhibitory complex.

In light of this model, we asked the following questions: (1) How does phosphorylation stabilize the Swe1/Cdk complex? (2) How does complex formation affect the kinetics of Swe1 phosphorylation by Cdk1, and Cdk1 phosphorylation by Swe1? and (3) How does hyperphosphorylation lead to complex dissociation? This line of inquiry led us to investigate the Cdk-subunit and phosphate-binding protein Cks1. We refocused and expanded our questions to determine the roles of Cks1 in multisite phosphorylation of Swe1 (and other substrates) by Cdk.

1.4. Approach

We asked these questions as biochemists and structural biologists, and therefore we sought answers in terms of quantitative parameters and detailed molecular models of the protein-protein interactions involved. Our preferred

strategy is to attempt to reduce a complex system to one of as few components as necessary to recapitulate the phenomena under investigation. This means that often we are using truncated or modified constructs of proteins that never actually appear *in vivo*. Toward our ultimate goal of understanding the highly multiphosphorylated substrate Swe1, we switched to studying a smaller, less highly phosphorylated Cdk1 substrate, Cdc6. We also perform experiments in artificial conditions very different from the cellular environment, separate from all the other kinases, substrates, phosphatases, and thousands and thousands of other molecules normally present and dynamic within any living system. We study yeast proteins with the knowledge that yeast itself is a simple yet imperfect model of human cells.

1.5. Cyclin-Dependent Kinases and Cks Proteins

This dissertation is largely focused on the question of how Cks proteins affect Cdk activity, but it is useful to recall other mechanisms of Cdk regulation for purposes of comparison. A summary of *S. cerevisiae and H. sapiens* cyclins, Cdks, Cks proteins and Cdk regulators is given in Table 1.1. Cyclin-dependent kinases consist of a catalytic serine/threonine kinase subunit that is activated by binding to a regulatory cyclin subunit. In accordance with their crucial role in the execution of cell cycle events, Cdk activity is highly regulated. Cyclins not only activate Cdks in a periodic manner, but also govern substrate selection in order to promote cell-cycle stage specific events. Cdks may also be regulated by both activating and inhibitory phosphorylation. Full activation of Cdks usually

Table 1.1. Table of Cell Cycle Regulators. Cyclin-dependent kinases and a subset of their regulators are listed for the model organisms most relevant to this dissertation. The preferred nomenclature for budding yeast Cdc28 and fission yeast Cdc2 homologs is now Cdk1. While human Cks homologs are referred to in some references as “Cks1” and “Cks2,” within this dissertation the human Cks proteins will be referred to as CksHs1 and CksHs2 for clarity.

Species	Cyclin (Cdk)				Cks Proteins	Inhibitory Kinases	Inhibitors
	G1	G1/S	G2	M			
S. cerevisiae	Cln3 (Cdk1)	Cln1,2 (Cdk1)	Clb5,6 (Cdk1)	Clb1,2,3,4 (Cdk1)	Cks1	Swe1	Sic1, Far1
S. pombe	Puc1? (Cdk1)	Puc1, Cig1? (Cdk1)	Cig2, Cig1? (Cdk1)	Cdc13 (Cdk1)	p13suc1 (suc1)	Wee1, Mik1	Rum1
X. laevis		E (Cdk2)	E, A (Cdk2, 1)	A, B (Cdk1)	p9 (Xe-p9)	Wee1, Myt1	Xic1
H. sapiens	D1,2,3 (Cdk4,6)	E (Cdk2)	A (Cdk2,1)	B (Cdk1)	CksHs1, CksHs2	Wee1, Myt1	Cip/Kip (p21, p27, p57) INK4 (p15, p16, p18, p19)

requires phosphorylation by Cdk-activating kinase, CAK, at a specific threonine near the active site. Inhibitory phosphorylation on a conserved tyrosine (and in vertebrates, an adjacent threonine) by Wee1 and other kinases is an important mechanism for coordinating growth and division at entry into S phase and M phase (Kellogg 2003). Cdks are also inhibited by the binding of inhibitory proteins, which differ between species but share similar functions. Finally, the most poorly understood mechanism of Cdk regulation is association with Cks proteins. Cks proteins are small (9-18 kDa), evolutionarily conserved (Figure 1.1), noncatalytic proteins that physically associate with Cdks. The following sections review the history of Cks protein research from their discovery in 1986 to the present.

1.5.1. Cks Proteins: the '80's

Cks was originally discovered in *Schizosaccharomyces pombe* as a suppressor of a *cdc2* mutant (Hayles et al. 1986). The authors called the gene *suc1* because it was a multicopy *su*ppressor of *c*ell cycle block caused by inactive *cdc2* gene product, which we now know to be Cdk1. Subsequent work in the Nurse lab showed that *suc1* does not operate through changes in the transcription levels of *cdc2*, supporting evidence for a physical interaction between their gene products (Hayles, Aves, and Nurse 1986). Soon after, the *suc1* gene product $p13^{suc1}$ was cloned and expressed in *E. coli* where it was discovered

Figure 1.1. Sequence Alignment of Cks Proteins. Darker boxes represent higher conservation. Alignment was performed with T-COFFEE and image generated using BOXSHADE. The hinge region motif HXPEPH is highly conserved, as are the anion binding pocket residues R33, R42, S82, W85, and R102(*S. cerevisiae* numbering).


S.cer 1 MYHHYHAFQGRKLTDQERARVLEFQDSIHYSPRYSDDNYEYRHVMLPKAMLKVIPSDYFN
S.pom 1 MSKSG---VPRLLTASERERLEPFIDQIHYSPRYADDEYEYRHVMLPKAMLKAIPTDYFN
H.sap1 1 MSH----------KQIYYSDKYDDEEFEYRHVMLPKDIAKLVPKT---
H.sap2 1 MAH----------KQIYYSDKYFDEHYEYRHVMLPRELSKQVPKT---
X.lae 1 MSY----------KNIYYSDKYTDEHFEYRHVMLPKELAKQVPKT---
C.ele 1 MTTG----------NNDFYYSNKYEDDEFEYRHVHVTKDVSKLIPKN---
S.cer 61 SEVGTLRILTEDEWRGLGITQSLGWEHYECHAPEPHILLFKRPLNYEAELRAATAAAQQQ
S.pom 58 PETGTLRILQEEEWRGLGITQSLGWEMYEVHVPEPHILLFKREKDYQMKFS---------
H.sap1 36 ------HLMSESEWRNLGVQQSQGWVHYMIHEPEPHILLFRRPLPKKP----------
H.sap2 36 ------HLMSEEEWRRLGVQQSLGWVHYMIHEPEPHILLFRRPLPKDQ----------
X.lae 36 ------HLMSEEEWRRLGVQQSLGWVHYMIHEPEPHILLFRRPLPKDQ----------
C.ele 38 ------RLMSETEWRSLGIQQSPGWMHYMIHGPERHVLLFRRPLAATQK----------
S.cer 121 QQQQQQQQQQQQQHQTQSISNDMQ--VPPQIS
S.pom 109 ----------Q----------QRGG
H.sap1 78 ----------KK
H.sap2 78 ----------QK
X.lae 78 ----------QK
C.ele 81 ----------TGGNVRSGNAVGVR

that “a protein of approximately 13 kd accumulated massively following IPTG induction” (Brizuela, Draetta, and Beach 1987). Antibodies generated against $p13^{suc1}$ were used to determine that $p13^{suc1}$ levels do not vary throughout the cell cycle and $^{32}PO_4$ kinase assays showed that $p13^{suc1}$ was unlikely to be a Cdk substrate (Brizuela, Draetta, and Beach 1987). Brizuela et al. (1987) also showed that the *S. pombe* Cdk $p34^{cdc2}$ and $p13^{suc1}$ physically associate and that the $p34^{cdc2}/p13^{suc1}$ complex retains enzymatic activity. Not until 1989 was the budding yeast homolog of *suc1* discovered in a similar high-copy suppressor screen for genes that rescue a temperature-sensitive *cdc28* mutation (Hadwiger et al. 1989). The suppressor was designated *CKS1* for *cdc28 kinase subunit* 1 and the Cks1 protein was found to associate with Cdc28 (Hadwiger et al. 1989).

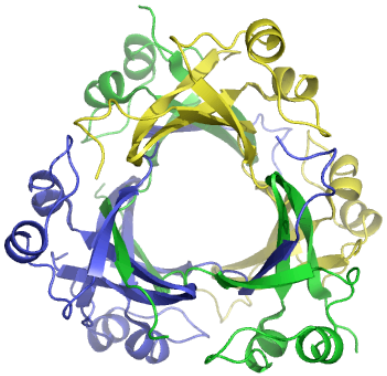
Given its ease of production, stability, and conservation, $p13^{suc1}$ became a valuable tool for isolation of *cdc2* homologs in other organisms. In the mid-1980s, purification of M-phase promoting factor (MPF) from *Xenopus* oocyte extracts was arduous and its components were unknown, as was the relationship of MPF to the *cdc* gene products (Gerhart et al. 1985). In 1988, a heroic six-step chromatography scheme identified two proteins, a 45-kDa protein and a 32-kDa protein, that co-eluted with MPF activity and protein kinase activity (Lohka, Hayes, and Maller 1988). Taking advantage of the interaction between $p13^{suc1}$ and $p34^{cdc2}$, Dunphy and colleagues used $p13^{suc1}$ as a purification reagent and determined that the *Xenopus* homolog of *cdc2* is in fact the 34-kDa component of MPF (Dunphy et al. 1988). The MPF components purified by Lohka et al. 1988

and the 34-kDa and 42-kDa proteins that specifically bound p13-agarose were strikingly similar. In 1990, the cDNAs for two mammalian *suc1*/*CKS1* homologs were cloned and found capable of substituting for *CKS1* in budding yeast and binding $p34^{cdc2}$ or Cdc28 kinase—strong evidence for the universal conservation of eukaryotic cell cycle control mechanisms (Richardson et al. 1990). Altogether, Cks proteins made an invaluable contribution to the famous confluence of genetic and biochemical approaches that allowed biologists to define Cdks as the universal engines of the cell cycle.

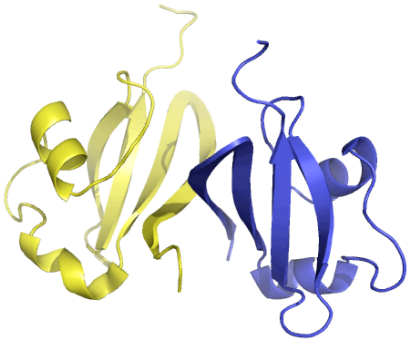
1.5.2. Cks proteins: the '90's

The story of Cks proteins is a parable of how X-ray crystallographic analysis can spur hypotheses both right and wrong. (An assortment of Cks structures is shown in Figure 1.2.) Genetic and biochemical analysis of Cks proteins and their relationship to Cdks continued into the early 1990s, but was confounded by the accumulation of contradictory evidence as to the function of Cks proteins. In a *cdc2* mutant background, *suc1* overexpression can rescue arrest at both G1/S and G2/M, but in wild-type cells overexpression causes arrest (Brizuela, Draetta, and Beach 1987). G2 arrest is caused by p13suc1 overexpression in fission yeast but by defective Cks1p in budding yeast (Hayles, Aves, and Nurse 1986; Y. Tang and Reed 1993). The first crystal structure of a Cks protein was that of human CksHs2 in 1993, and it generated a flurry of creative yet ultimately refuted hypotheses (Parge et al. 1993). CksHs2 (also called $p9^{CKS2}$) crystallized in a hexameric assembly consisting of three strand-exchanged

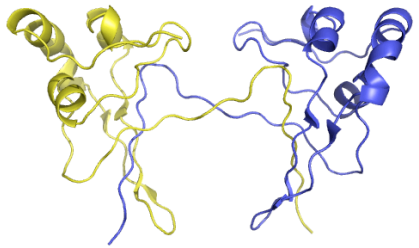
Figure 1.2. X-ray Structures of Cks Proteins. Cks proteins have a conserved overall architecture consisting of four β-strands and 2-3 α-helices. CksHs2 and suc1 have crystallized in both monomer and domain-swapped folds. CksHs1 has only crystallized as a monomer, and wild-type Cks1 has only crystallized as a domain-swapped dimer. Cks proteins = yellow, blue, green; Cdk2 = violet; p27 phosphopeptide = orange; Skp1 = grey; Skp2 = red.



CksHs2
1CKS
1993

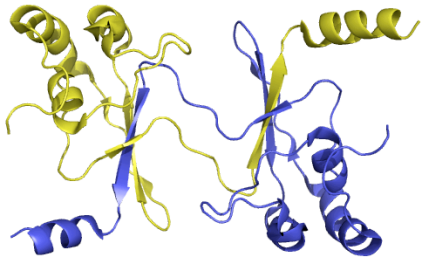


CksHs1
1DKS
1995

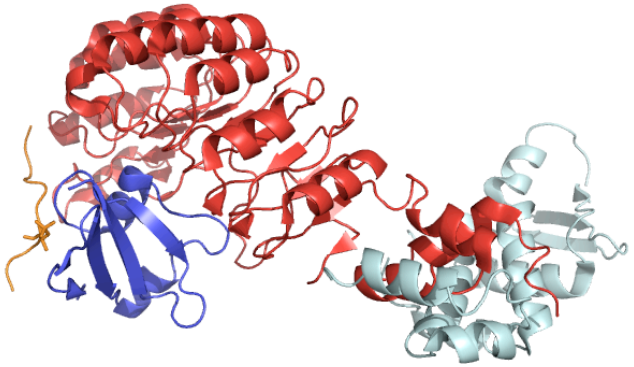


suc1
1PUC
1996

Cdk2/CksHs1
1BUH
1996



Cks1
1QB3
2000



Skp1/Skp2/CksHs1/p27
2AST
2005

dimers. This appears to have surprised and challenged the authors, whose "numerous attempts" to fit a monomeric subunit model of CksHs2 into the electron density were unsuccessful (Parge et al. 1993). The authors acknowledge that the presence of Pro62 and Pro64, two conserved hinge-region prolines between strands β3 and β4, suggest a likely β hairpin, but are unable to refine such a model. Instead, the first glimpse of Cks protein structure revealed a novel threefold symmetric 12-stranded anti-parallel β barrel motif that was assumed to be biologically relevant because the residues involved were evolutionarily conserved (Parge et al. 1993). By gel filtration, CksHs2 behaved as a mixture of monomers, dimers, and hexamers. Nevertheless, the idea of CksHs2 hexamers as a hub for multimeric assembly and localization of Cdks was attractive. Not until the structure of CksHs1 bound to Cdk2 was solved (also by the Tainer group) in 1996 did it become clear that strand exchange was incompatible with Cdk binding (Bourne et al. 1996).

Another idea that arose from early Cks structures was that multimerization was related to metal/ion chelation. Hexamer formation was apparently favored in CksHs2 crystal conditions due to a high concentration of sulfate (Parge et al. 1993). In 1995, the structure of $p13^{suc1}$ was solved as a non-strand exchanged dimer, the formation of which was highly dependent on the presence of zinc ions (J. A. Endicott et al. 1995). Zinc ion chelation was found to be incompatible with Cdk binding, suggesting a mechanism for zinc-mediated Cdk regulation via Cks proteins (Morris, Heitz, and Divita 1998). Strand-

exchanged dimers of $p13^{suc1}$ with a fold resembling that of dimeric CksHs2 were also inducible and amenable to crystallography, generating interest in the dynamics and conformational properties of Cks proteins independent of their role in cell cycle control (Birck et al. 1996; Bourne et al. 1995).

The growing list of Cks1/$p13^{suc1}$ homologs provided information about evolutionarily conserved regions of Cks proteins, generating new functional hypotheses. Cks proteins appeared to contain two clusters of conserved amino acids. In addition to the hinge-region dual proline motif, which was presumed to play a functional role in oligomerization, Cks proteins also possess a positively charged cluster of residues. In the hexameric assembly of CksHs2, this cluster was found bound to sulfate ion, raising the possibility that this pocket may bind phosphate and, by extension, phosphoproteins. Combined with evidence that $p13^{suc1}$ interferes with the activating dephosphorylation of $p34^{cdc2}$ at mitotic entry in *Xenopus* extracts, the presence of a phospho-binding pocket suggested Cks proteins may bind Cdk via its inhibitory phosphotyrosine and protect Cdk from dephosphorylation by Cdc25 (W G Dunphy and Newport 1989). Even after the structure of Cdk2 bound to CksHs1 was solved, this hypothesis persisted, as an explanation was sought for the ability of $p13^{suc1}$ to block Cdk dephosphorylation. The idea that more than one Cks monomer could bind Cdk at a time—one binding in the manner observed in the crystal structure, another blocking access to phosphotyrosine—was called in one review a "speculative explanation with no supporting data" (Pines 1996). That $p13^{suc1}$ was capable of

binding active $p34^{cdc2}$ was considered evidence that there are two binding sites on Cdk for Cks proteins (J. A. Endicott and Nurse 1995). In light of what we now know, a more plausible explanation is that Cks-phosphoCdc25 interaction is an important mechanism for targeting Cdc25 to pTyr-Cdk, and excess free Cks1 interferes with the ability of phosphoCdc25 to bind Cdk.

The 1996 structure of CksHs1 bound to Cdk2 resolved many unanswered questions about Cks-Cdk interaction. For one, previous attempts to locate the Cks binding site on Cdk using alanine scanning mutagenesis had suggested a requirement for two separate domains of Cdk (Ducommun, Brambilla, and Draetta 1991). The Cdk2/CksHs1 structure showed that only one Cdk2 domain, the C-terminal lobe, was directly involved in CksHs1 binding (Bourne et al. 1996). Notably, the phosphate anion-binding site was not involved with Cdk binding but was instead solvent-exposed on the same surface as the Cdk catalytic site, thereby providing an extended surface for substrate recognition. From this important structure came the idea that Cks proteins direct Cdks to phosphorylated substrates or other regulatory proteins, an idea that continues to garner experimental support to this day.

Some of the earliest experimental support for the substrate-targeting model of Cks-mediated Cdk regulation came from experiments performed in *Xenopus* oocyte extracts in the late 1990s by the Dunphy group. These experiments also confirmed that, in contrast to stage-specific cyclins or checkpoint regulators, Cks proteins have multiple essential roles in the cell cycle,

just like Cdk itself. Immunodepletion of the *Xenopus* Cks protein Xe-p9 from interphase extracts prevents mitotic entry because Cdc2 dephosphorylation by Cdc25 is inhibited (Patra and Dunphy 1996). If these extracts are driven into mitosis using a constitutively active Cdc2 mutant Cdc2-AF, however, they encounter another barrier: mitotic exit is stalled because cyclin B degradation is defective (Patra and Dunphy 1996). This result suggests that Cks proteins may interact with the anaphase-promoting complex (APC), which is responsible for targeting cyclin B for degradation at mitotic exit. Since several APC subunits are hyperphosphorylated by CycB/Cdc2 in mitosis, Patra and Dunphy hypothesized that Xe-p9 might be important for APC subunit phosphorylation, and thus for the activation of the APC (Patra and Dunphy 1998). When Xe-p9 immunodepleted extracts were driven into mitosis using Cdc2-AF and the phosphorylation state of APC subunit Cdc27 was observed, Cdc27 was no longer extensively hyperphosphorylated, an effect that correlated with stabilization of cyclin B (Patra and Dunphy 1998). This was the first time that a Cks protein was shown to be important in phosphorylation of a Cdk substrate. Dunphy and colleagues went on to show that Xe-p9 is required for full phosphorylation of a number of highly phosphorylated mitotic Cdk substrates, including mitotic regulators Wee1, Cdc25, and Myt1 (Patra et al. 1999). Roles for Cks proteins in APC activation and hyperphosphorylation of Cdk substrates were confirmed in multiple model organisms, and Cks proteins proved a useful affinity reagent for active APC purification as well (Rudner and Murray 2000; Sudakin 1997). The Deshaies

group showed that Cks1 is also important for phosphorylation of G1 Cln-Cdk substrates Sic1 and Far1 (Reynard et al. 2000). *C. elegans* possess two Cks homologs that participate in Cdk inactivation and cyclin degradation in anaphase (Polinko and Strome 2000). In clam oocyte extract, clam Suc1 stimulates APC activation and the hyperphosphorylation of a number of proteins, although not all phosphoproteins, in the extract (Shteinberg and Hershko 1999). Shteinberg and Hershko's results were the first to indicate that specificity of Cks-dependent substrate targeting might be more complex than minimal phosphoSer/Thr recognition. More recent research shows that in humans, CycB/Cdk1/Cks1 is recruited to the APC as a substrate via the phosphorylated Cdc27 homolog APC3 binding to Cks1 (van Zon et al. 2010).

1.5.3. Cks Proteins: the 21st Century

Much of the research on Cks proteins in the last decade has focused on questions of the folding and dynamics of domain swapping. While many Cks dimerization questions seem to be motivated by interest in the potential for biological relevance, the conclusions generated tend not to support the existence of Cks monomer-dimer equilibrium *in vivo*. Domain-swapped dimerization is interesting in its own right as a protein folding problem: how does a single primary sequence encode for two unique stable tertiary structures? By what mechanisms does monomer-dimer interconversion occur? What intrinsic properties of a protein, domain, or region enable domain swapping? Cks proteins are an excellent system to investigate these questions because they are small,

extremely stable, easily expressed, and evolutionarily conserved. The small size of Cks proteins simplifies the process of identifying the contributions of individual domains and residues through protein engineering. Static crystal structures were complemented by experiments measuring the unfolding kinetics and thermodynamics of various Cks mutants, usually in $p13^{suc1}$ (which was by this time usually referred to as "suc1"), demonstrating that suc1 monomer-dimer transitions occur via an unfolded intermediate and were unlikely to occur spontaneously *in vivo* (Schymkowitz et al. 2000). Later it was confirmed that human CksHs1 and CksHs2 also transit through an unfolded intermediate upon dimerization (Seeliger et al. 2002). Of special interest were the conserved prolines in the hinge-region (HXPEPH) between the β3 strand and the exchanging β4 strand. Alanine substitution at the second proline has no effect on the thermodynamic stability of suc1, while alanine substitution at the first proline stabilizes suc1, suggesting that because proline is conserved at this position despite an energetic cost, it is likely to have an important structural or functional role (Schymkowitz, Rousseau, and Itzhaki 2000). The apparent storage of energy in the hinge region was referred to as a "loaded molecular spring" that relieves tension by adopting the dimer conformation (Rousseau et al. 2001). Further work from the Itzhaki group showed that partitioning between monomer and dimer was entirely controlled by the two hinge region prolines (Rousseau et al. 2001). To determine the contribution of regions outside of the hinge loop to monomer-dimer equilibrium, thirty of suc1's 113 residues at

various positions within the protein were mutated and the K_d and $\Delta\Delta G_{dissociation}$ of dimeric suc1 were calculated (Schymkowitz et al. 2001). Almost every position had an effect on the K_d of the dimer, which the authors interpreted as evidence that hinge-region-generated strain is actually distributed throughout the protein (Schymkowitz et al. 2001). That sites distant from the hinge region can affect domain swapping suggests that signal transduction might occur via strain propagation within Cks—for example, ligand binding at the anion-binding site could be communicated to the Cdk binding site, or vice versa. The authors propose that signal transduction is a more likely explanation for the conservation of hinge-region strain and, by extension, dimerization propensity, than a biological role for dimeric suc1 (Schymkowitz et al. 2001). In Chapter 3 I will argue that the existence of Cks signal transduction is not required to make the case that the adaptor function of Cks proteins is sufficient to explain their conserved structural and dynamic properties.

As the idea took root that Cks proteins function as phosphoprotein adaptor proteins, scientists wondered whether phosphate binding was connected to the oligomerization state of Cks. Since the anion-binding pocket is composed of residues from all four β strands, including the swapped strand, some type of structural stabilization effect seems likely. Schymkowitz et al. (2001) found that upon addition of phosphate or cdc25 phosphopeptide to wild-type suc1, the monomer form was stabilized relative to the dimer. However, a separate study found that wild-type suc1 has a 5-fold higher affinity for the same

cdc25 phosphopeptide than a suc1 P90A mutant, which is preferentially monomeric (Odaert et al. 2002). This result was one of the first indications that wild-type monomer and proline-mutant monomer are non-identical beyond the single amino acid substitution.

In the last decade our understanding of Cks proteins in mammalian systems and cancer biology has greatly increased. High Cks expression is correlated with many cancers, including lung, oral, colorectal, ovarian, prostate, and breast cancers (Inui et al. 2003; Kitajima et al. 2004; Shapira et al. 2004; Yamamoto et al. 2009; Lan et al. 2008; Westbrook et al. 2009). Low Cks1 expression is correlated with increased survival in patients with squamous cell lung cancer independent of tumor stage, and overexpression of Cks1 is strongly associated with poor prognosis in breast, liver, and salivary cancers (Zolota et al. 2010; X.-C. Wang et al. 2009; Shen et al. 2010; Nagler et al. 2009). In cancer cell culture models, overexpression of CksHs1 or CksHs2 confers the ability to evade the DNA damage checkpoint response (V. Liberal et al. 2011). Therefore, Cks may be an important prognostic marker of cancer and a potential therapeutic target.

An important function for Cks proteins that appears to be peculiar to mammalian Cks and CksHs1 in particular is p27 degradation. CksHs1 is accessory protein in the SCF^{Skp2} ubiquitin ligase complex that is essential for *in vitro* reconstitution of p27 ubiquitination using purified components (Ganoth et al. 2001). CksHs1 is important for p27 ubiquitination because it associates with SCF component Skp2 and binds to p27 that is phosphorylated on T187, a

modification that was known to be necessary for recognition of p27 by SCF^{Skp2} and p27 degradation (Vlach, Hennecke, and Amati 1997; Carrano et al. 1999). CksHs1 is able to function in this capacity independently of Cdk (Spruck et al. 2001).

The structural basis of the requirement for CksHs1 in p27 recognition was discovered through the X-ray crystal structure of Skp1/Skp2/CksHs1 bound to a phosphoT187 p27 peptide (Hao et al. 2005). The phosphate of p27 binds in the anion-binding pocket of CksHs1. Grant reviewers have sometimes referenced this structure as evidence that Cks-phosphoprotein binding specificity is likely to be low, given that the only p27 residue involved in CksHs1 binding is phosphoT187 (Hao et al. 2005). The ubiquitin ligase function of CksHs1 may be unique, as CksHs2 is not capable of substituting for CksHs1 in Skp2 binding (Ganoth et al. 2001). One hypothesis might be that in mammals, CksHs2 and CksHs1 have diverged functionally such that CksHs2 is the more Cks1/suc1-like of the homologs, while CksHs1 evolved for an SCF-substrate recognition role. However, the high structural and sequence conservation between CksHs2 and CksHs1 makes this a difficult point to argue.

1.6. Cdk Substrates

Sic1, Cdc6, and Swe1 are all multiply phosphorylated Cdk substrates that also act as Cdk inhibitors. For each of these substrates, I will summarize their relevance to this dissertation, their basic biology, and the current state of knowledge relating to their phosphoregulation by Cdks.

1.6.1. Swe1

As explained above, our investigation into multiphosphorylated Cdk substrates in budding yeast began with Swe1. Unfortunately, most of the questions we asked when we embarked remain unanswered. My hope is that the contributions within this dissertation increase our ability to understand and experimentally approach these questions, should some intrepid scientist choose to return this project to its "Swe roots."

Swe1 is the budding yeast homolog of wee1, which was originally identified as a negative regulator of mitosis in fission yeast (Paul Russell and Nurse 1987). That *wee1*$^{-}$ yeast entered mitosis early and therefore at a smaller size earned the gene its name and established wee1 as a key regulator of the cell size checkpoint. Swe1 and its orthologs are tyrosine kinases that regulate the cell size checkpoint by phosphorylating and inhibiting M-phase cyclin-Cdks until a critical size is reached. Swe1 and its homologs are extensively phosphorylated in mitosis (Kellogg 2003). Harvey et al. (2005) showed that Swe1 is a Clb2/Cdk1 substrate, that Swe1 is first activated and then inactivated by Clb2/Cdk1 phosphorylation, that Clb2/Cdk1 phosphorylation of Swe1 is necessary for the formation of a stable Swe1/Clb2/Cdk1(/Cks1) complex prior to mitotic entry, and that formation of this complex is essential for the maintenance of Cdk1 inhibition prior to mitosis. The Kellogg lab in collaboration with Steven Gygi learned that Swe1 is phosphorylated at 8 of 13 Cdk consensus sites (S/T-P) and 10 nonconsensus sites (S/T), with nonconsensus phosphorylation depending on

prior phosphorylation at consensus sites. This particular aspect of Swe1 regulation led our research questions toward Cks1 and the priming model hypothesis for Swe1 hyperphosphorylation. All 18 mapped Cdk1 phosphorylation sites are in the natively unfolded N-terminal domain of Swe1. The NTD of Swe1/Wee1 is highly evolutionarily divergent in sequence, but the presence of multiple (5+) phosphorylation sites is conserved, suggesting a position-independent function for hyperphosphorylation (Kim and Ferrell 2007).

1.6.2. Cdc6

Cdc6 was an attractive protein for our studies for multiple reasons. First, like Swe1, it possesses a multiply phosphorylated, natively unstructured N-terminal domain. Second, also like Swe1, it has been shown to form a stable, phosphorylation dependent complex with Clb2/Cdk1. Third, in contrast to Swe1, the NTD of Cdc6 has only four consensus Cdk sites and no documented nonconsensus phosphorylation. For these reasons, we considered Cdc6 a simplified model substrate with which to identify Cks1 interactions that might be harder to tease out using Swe1.

Cdc6 is an essential protein in DNA replication in *S. cerevisiae* and many other organisms, including humans. Cdc6 promotes assembly of the multicomponent prereplicative complex (preRC) at replication origins (Bell and Dutta 2002). PreRC assembly can only occur in the absence of CDK activity, or from mitotic exit to early G1 (Weinreich et al. 2001). In the presence of CDK

activity, such as in late G1, Cdc6 is multiphosphorylated and targeted for ubiquitylation and rapid degradation by the E3 ubiquitin ligase SCF^{CDC4} (Perkins, Drury, and Diffley 2001). Degradation of Cdc6 prevents reassembly of the preRC and thus prevents refiring of replication origins, ensuring that DNA replication occurs once and only once per cell cycle.

Cdc6 possesses eight Ser-Pro or Thr-Pro Cdk consensus motifs and six preferred Cdk consensus sites (S/T-P-X-K/R). The 47-amino acid N-terminal domain (NTD) of Cdc6 is predicted to be natively unfolded and contains four Cdk phosphorylation sites, three of which are preferred consensus sites. In addition to promoting Cdc6 degradation, Cdc6 phosphorylation promotes binding to Clb2-Cdk complexes, inhibits Cdc6 nuclear localization, and prevents Cdc6 from associating with chromatin (Mimura et al. 2004; Honey and Futcher 2007). Together these effects of phosphorylation help prevent Cdc6 reloading and DNA rereplication. Cdc6 has also been implicated in CDK inhibition at mitotic exit. Inactivation of CDKs is required for exit from mitosis and involves both Cdk inhibition and active degradation of B-type cyclins. Cdc6 has been shown to form phosphorylation dependent stable complexes with Clb2-Cdk during late mitosis, contributing to mitotic exit (Calzada et al. 2001). The Cdc6 NTD not only contains a Cdc4-binding phosphodegron, but also a phosphorylation dependent Cdk-interacting domain (Mimura et al. 2004).

1.6.3. Sic1

Sic1 is a superstar in the field of multisite phosphorylation. Sic1, like Swe1 and Cdc6, contains a natively unfolded N-terminal domain with multiple Cdk phosphorylation sites. In budding yeast, Sic1 is a CDK inhibitor in G1 that is targeted for degradation upon phosphorylation by Cln-Cdks when cells commit to Start (Schneider, Yang, and Futcher 1996). While the NTD of Sic1 contains nine Cdk consensus sites, only a subset need be phosphorylated to constitute a phosphodegron recognizable by SCF^{CDC4} ubiquitin ligase (Nash et al. 2001). Nash et al. (2001) determined that six Cdk sites were necessary for Sic1 recognition by Cdc4. One playfully written review refers to a "six strips of Velcro" versus "one drop of superglue" model of Cdc4 phosphoSic1 recognition, indicating that while each individual phosphosite has relatively weak affinity for Cdc4 compared to an optimal phosphodegron, six weak binding sites function together to create a stable association (Deshaies and Ferrell Jr. 2001). A series of nuclear magnetic resonance studies explore the dynamic mode of binding available to the intrinsically disordered Sic1 NTD (Borg et al. 2007; Mittag et al. 2008; Mittag et al. 2010; Xiaojing Tang et al. 2012). While this mode of binding is fascinating from both a structural and a signaling perspective, recent evidence suggests that the cooperativity inherent in the all-or-nothing, switch-like destabilization of Sic1 may not dependent on dynamic interactions between Cdc4 and low affinity Sic1 phosphodegrons after all (Kõivomägi et al. 2011). Alternatively, together with our Estonian collaborators, we propose that the cooperativity in Sic1

degradation is generated by positive feedback between a first and second round of phosphorylation, where the second round is Cks1-dependent.

1.7. References

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Chapter 2: Defining the Kinetic Contribution of Cks1 to Cdk1 activity

2.1 Introduction

Phosphorylation is a mechanism for signaling at the level of protein-protein interaction. A protein that possesses multiple phosphorylation sites possesses great signaling potential when those sites are considered elements of a combinatorial logic scheme. Assuming a random phosphorylation mechanism, each site may independently occupy one of two discrete states (phosphorylated or unphosphorylated), allowing the generation of 2^n distinct phosphoforms for a protein with *n* phosphorylation sites, with *n*! possible pathways from unphosphorylated to fully phosphorylated (Salazar and Höfer 2007). In theory, each distinct phosphoform could produce a distinct output. Signaling via multisite phosphorylation may also follow a sequential logic scheme, in which not only the ON/OFF state of each site is considered information, but also the history of states that were occupied on the path from start to finish. In this case, the extent to which phosphorylation events are cooperative (versus independent), processive (versus distributive), or ordered (versus random) also determines the nature of the output.

Differences in K_m can have important consequences *in vivo*. Cdk1 is exposed to potential substrate concentrations of up to 1 mM in yeast (Loog and Morgan 2005). Clb2/Cdk1 exhibits a $K_m \sim 50$ μM toward a histone H1 peptide (considered a general substrate), while Clb5/Cdk1 is a less specific enzyme with $K_m \sim 500$ μM toward histone peptide. It has been proposed that this difference

allows Clb5/Cdk1 to "focus" on its highly preferred substrates first, despite the abundance of available Cdk phosphoacceptor sites early in the cell cycle.

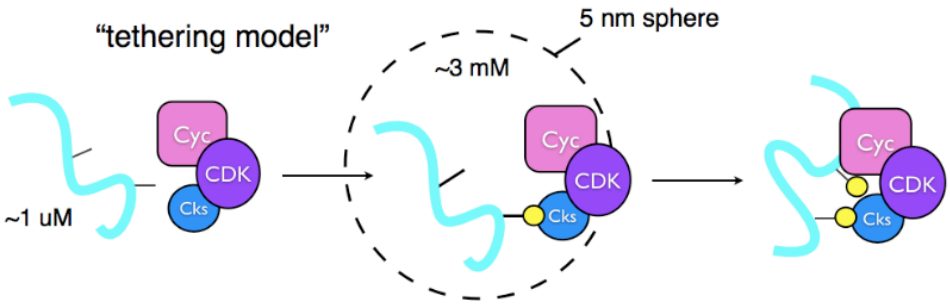


Figure 2.1. The Tethering Model of Cks1-Mediated Multisite Phosphorylation.

The tethering model of Cks1-mediated Cdk phosphorylation posits that preexisting phosphorylation on a multiphosphorylated Cdk substrate allows the substrate to dock to Cdk-associated Cks1, promoting further phosphorylation by bringing phosphoacceptor sites and the active site of the enzyme in close proximity for a longer period of time (Figure 2.1, adapted from Deshaies & Ferrell Jr., 2001). One prediction of our Cks1 tethering model is that the effective K_m of Cdk1 phosphorylation of Cks1-binding substrates will be lower than that of substrates that do not bind Cks1. Just as with cyclin-docking, this would have major implications for substrate preference of Cks1-bound Cdk versus Cks1-free Cdk. We hypothesize that Cks1 adds a layer of specificity to substrate selection in the mélange of the cellular milieu.

Cks1-stimulated phosphorylation is more complex than cyclin-stimulated phosphorylation because Cks1 does not bind substrate independently of

substrate phosphorylation state. Therefore there are many additional considerations when testing the tethering model of Cks1 substrate targeting experimentally. For example, a given substrate may possess more than one priming site. Phosphorylated substrates may bind Cks1 via an allovalent mechanism, increasing affinity for Cks1 with increasing phosphorylation (Levchenko 2003). Priming site binding may introduce acceptor site preference and therefore nonrandom phosphorylation order due to geometric constrains within the enzyme-substrate complex. Cks1 may increase processivity by decreasing the off-rate relative to k_{cat}, an effect that has been observed since the conclusion of our initial kinetic investigation (Kõivomägi, Valk, Venta, Iofik, Lepiku, Balog, et al. 2011). Cdk may experience increased product inhibition due to Cks1 binding fully phosphorylated substrates. Any of these factors may be occurring in combination with one another, to different extents in different substrates.

The simplest version of our model assumes that Cks1 only changes the affinity of Cdk for substrate via binding of a priming phosphate on the substrate. This means that Cks1 binding does not affect substrate preference at the Cdk catalytic site or intrinsic catalytic ability, and that cyclin preference is also unaltered by the presence of Cks1. This assumption results in the prediction that because the enzyme-substrate complex is stabilized but k_{cat} is unchanged, K_m will concomitantly decrease. Despite the complexity of the system, these assumptions allow a standard kinase assay to be used to measure K_m of Cdk1

phosphorylation. Our rationale was that regardless of confounding factors, a change in K_m would still be an observable and informative parameter.

This chapter begins with the expression, purification, and activation of milligram quantities of *S. cerevisiae* Clb2/Cdk1. It then describes the optimization of initial kinase assays using this kinase with Cdc6 NTD. The kinetics section concludes with final promising results with multiple Cdk substrates accompanied by an important caveat. Results also include mass spectrometry experiments that complement kinetic assays by looking at the site-specific contribution of Cks1 to Swe1 NTD phosphorylation. The discussion suggests possible explanations for the results we observed and considers the ways in which Cks1 might contribute to ultrasensitivity.

2.2. Results

The expression and purification of pure, active Clb2/Cdk1 was a substantial undertaking and we are somewhat exceptional for our successful results (Derek McCusker, personal communication). His_6-Cdk1 was expressed in Sf9 cells initially using a baculovirus constructed by Seth Rubin. Later a His_6-Cdk1 expression vector was created using the Bac-to-Bac system and its sequence was verified by Will Finch (see page 34 of Will's lab notebook). Initial expression levels were low and protein expression was confirmed by Western blot (Figure 2.2a). Optimal expression was eventually ~3 mg/L (Figure 2.2b). The factor with the greatest contribution toward optimal expression was infection at low-to-medium cell density relative to our standard protocol (~1.5 x

10^6 cells/ml instead of 2 x 10^6). Expression for 72 hours was an improvement over expression for 24 hours so cultures were allowed to grow for at least two days following infection. His_6-Cdk1 can be either directly purified using Ni^{2+} resin

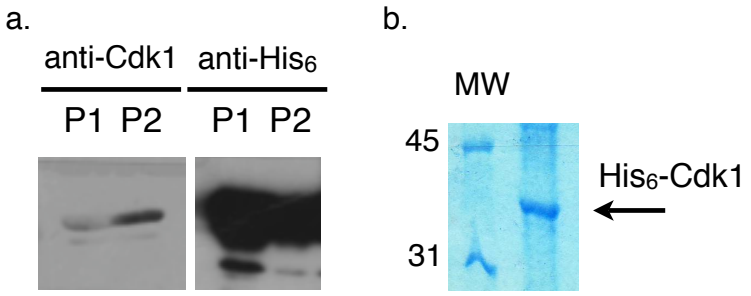


Figure 2.2. Optimizing the Expression of *S. cerevisiae* Cdk1. (a) His_6-Cdk1 expressed in *Sf9* cells with either P1 or P2 generation baculovirus was detected by Western blot. (b) Cdk1 expression visible by Coommassie stain following optimal expression.

or via its cyclin binding partner Clb2 (in the form of GST-Clb2). GST-Clb2 was expressed as a soluble fusion protein in *E. coli*. Approximately 20-40 mg of pure GST-Clb2 was immobilized to a column of 5-10 ml GS4B sepharose resin per 2L of Cdk-expressing culture, to ensure complete capture of His_6-Cdk1. Best results were achieved when GST-Clb2/His_6-Cdk1 complex was subsequently purified

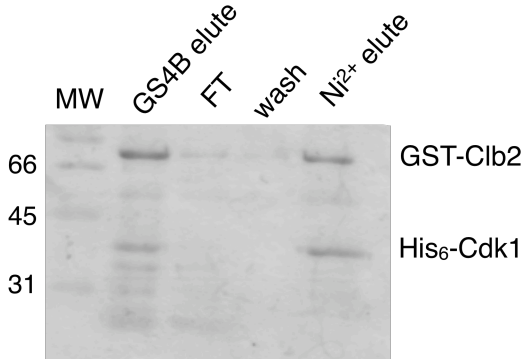


Figure 2.3. Tandem Purification of GST-Clb2/His_6-Cdk1. GST-Clb2 pulls down His_6-Cdk1 on GS4B resin. Assembled complex is then loaded onto a Ni^{2+} column and purified via Cdk1's His_6 tag.

using Ni^{2+} resin to ensure formation of Clb2/Cdk complex and remove nonspecific GS4B-binding *Sf9* proteins (Figure 2.3).

After removing the fusion tags by proteolytic cleavage, Clb2/Cdk1 was further purified by anion exchange on a 1 ml mono Q column. Clb2/Cdk1 may be activated by CAK prior to tag cleavage or anion exchange or afterwards with no apparent difference in activation efficiency, although this has not been extensively or quantitatively examined. If GST-CAK is used, it can be removed using GST-cleanup, which may be necessary following tag-cleavage. Phosphorylation by CAK activates Clb2/Cdk1, which can be monitored by radioassay due to Clb2/Cdk1 autophosphorylation (Figure 2.4a). CAK is not capable of phosphorylating Cdc6 NTD in the conditions of our assay, so contamination by 10% CAK was not considered problematic for kinetic experiments (Figure 2.4b).

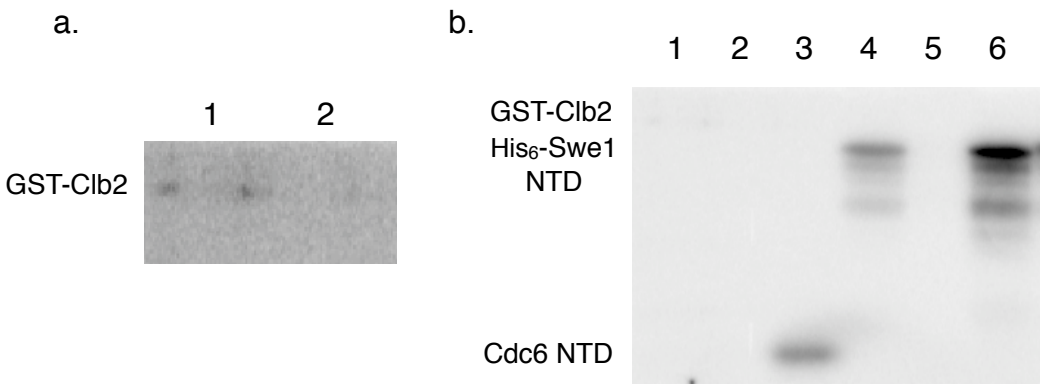


Figure 2.4. Autoradiographs Showing Clb2/Cdk1 Activity. (a) Adjusted exposure of lanes 1 & 2 of (b) (1) Clb2/Cdk1 autophosphorylates when CAK-activated. (2) Clb2/Cdk1 is inactive when unphosphorylated by CAK. (3) Cdc6 NTD and (4) Swe1 NTD are phosphorylated by CAK-activated Clb2/Cdk1 (5) Cdc6 NTD is not phosphorylated when CAK is present but Clb2/Cdk1 is absent. (6) Swe1 NTD phosphorylation by CycA/Cdk2, positive control.

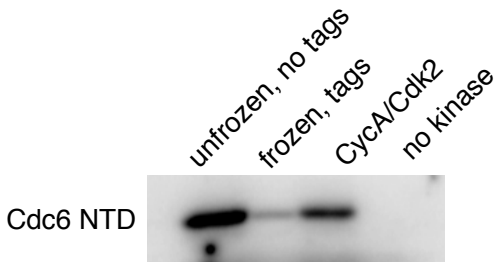


Figure 2.5. Assessing the Effects of Freezing on Cdk Activity. Here a frozen preparation of tagged GST-Clb2/His_6-Cdk1 shows reduced activity compared to tagless unfrozen Clb2/Cdk1. CycA/Cdk2 is used as a positive control.

Initial kinase assays were performed using Clb2/Cdk purified in the above manner, concentrated to 0.8 mg/ml + 10% v/v glycerol, aliquoted, and flash-frozen. There is some evidence of reduced activity of some preps upon freezing, although these differences may be attributable to the presence of fusion tags and/or prep-to-prep variability in activity (Figure 2.5). The strategy that best prevented reduced activity was to perform all purification, cleavage, and

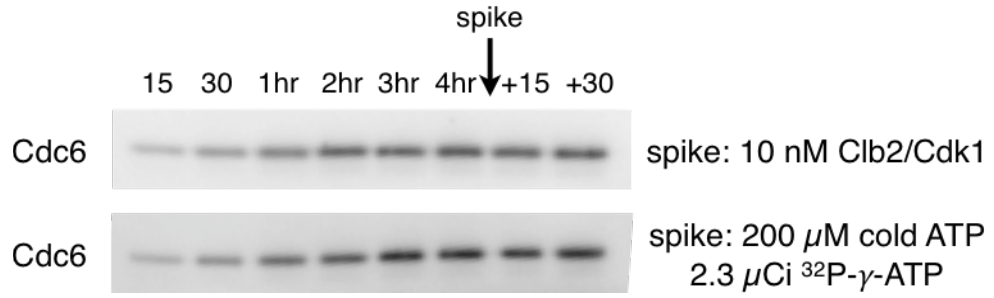


Figure 2.6. Autoradiograph of Saturation of Cdc6 Phosphorylation After Four Hours. 10 µl of 100 µl reactions were removed at each timepoint, quenched with 5 µl protein loading buffer, and 5 µl of each quenched mixture was resolved on a 15% polyacrylamide gel. After 4 hours, the remaining 40 µl was spiked with indicated quantities of fresh kinase or ATP to test whether kinase was unstable at room temperature or ATP supplies were limiting. Two subsequent timepoints were taken in which phosphate incorporation was stable, indicating that the reaction was not limited by active enzyme or ATP availability.

activating phosphorylation steps in as short a time frame as possible, without freezing protein in between.

A standard time course kinase assay of a multiply phosphorylated substrate does not reveal the kinetics of phosphorylation of individual sites, because the substrate migrates as a single band and so all incorporated ^{32}P is summed together. It does, however, tell us an effective initial rate of phosphorylation (v_0) that would be expected to increase if one or more sites are more efficiently phosphorylated in the presence of Cks1. The determination of v_0 requires information from the linear phase of the phosphorylation time course.

It is easiest to confirm that one is within the boundaries of the linear phase if complete phosphorylation (saturation) has occurred. It was determined that four hours (240 minutes) was sufficient to fully phosphorylate 1 μM Cdc6 (Figure 2.6). However, it should be mentioned that while the reaction may have terminated, no quantification of ^{32}P incorporation, such as using ATP standards,

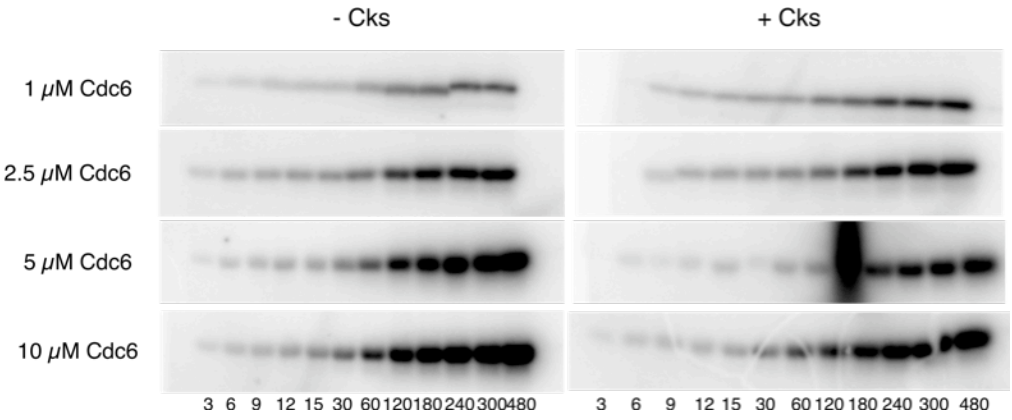
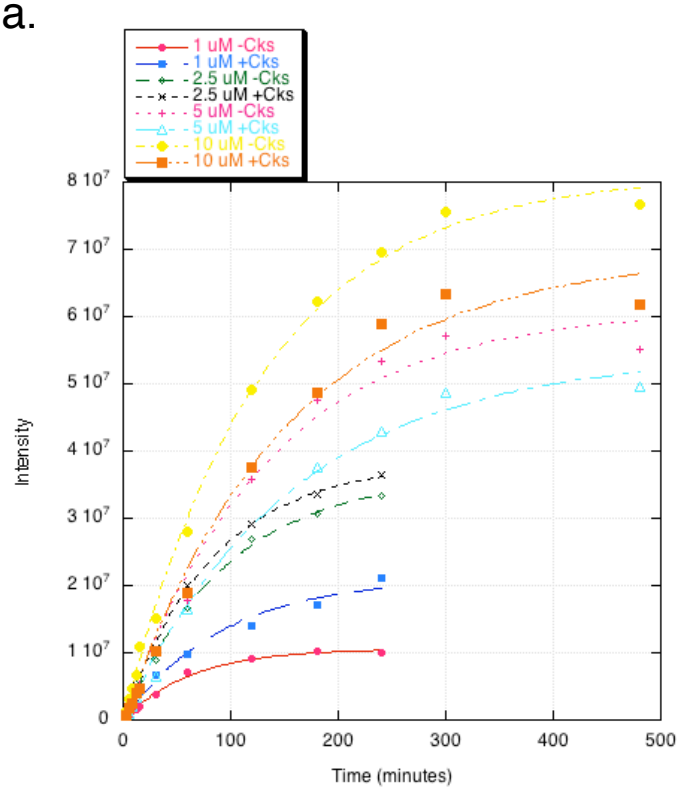


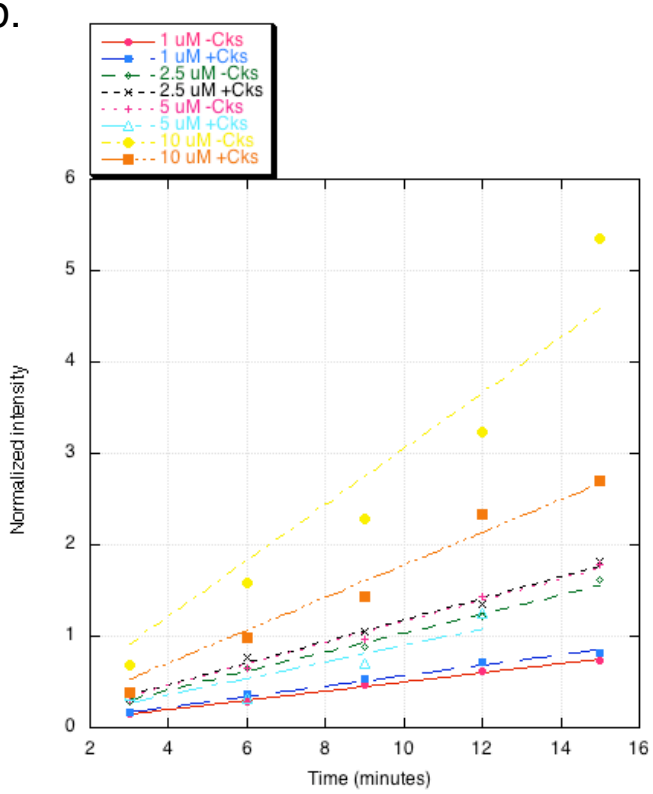
Figure 2.7. Time Course Phosphorylation of Cdc6 by Clb2/Cdk1 ± Cks1. 10 μl of kinase reactions performed at different Cdc6 concentrations was removed at each time point and quenched with 5 μl protein loading buffer. 5 μl of each quenched sample was loaded.

Figure 2.8. Representative Kinase Assay Results. (a) Total intensity within each band was background corrected and plotted as a function of time for each experiment. Curve fitting was performed using the first-order rate equation $y = m_1(1 - e^{-m_2 x})$ forcing a fit through the origin. Values for m_1 were defined as the extrapolated intensity of fully phosphorylated substrate in each reaction. Intensities were then normalized by multiplying by the total concentration of phosphoacceptor sites per reaction and dividing by m_1. Curve fitting and graph generation were done using KaleidaGraph. (b) The first five time points of kinase reactions were defined as the linear range and fit to a linear equation to determine v_0 in units of micromoles of phosphorylated residues per minute.

a.


1 uM -Cks
1 uM +Cks
2.5 uM -Cks
2.5 uM +Cks
5 uM -Cks
5 uM +Cks
10 uM -Cks
10 uM +Cks
Intensity
Time (minutes)

b.


1 uM -Cks
1 uM +Cks
2.5 uM -Cks
2.5 uM +Cks
5 uM -Cks
5 uM +Cks
10 uM -Cks
10 uM +Cks
Normalized intensity
Time (minutes)

was performed. Various concentrations of His_6-Cdc6 were phosphorylated over the course of an eight-hour (480 minute) time course (Figure 2.7 and Figure 2.8A). The first five timepoints, up to 15 minutes, were included in calculation of v_0 (Figure 2.8B). The expected differences in K_m or V_{max} were not observed in the presence of Cks1 (Figure 2.9). The error in curve fitting prevented the calculation of reliable K_m and V_{max}.

We asked whether Cks1-dependent effects were not observed because Clb2/Cdk1/Cks1 was not assembled as a ternary complex under the conditions

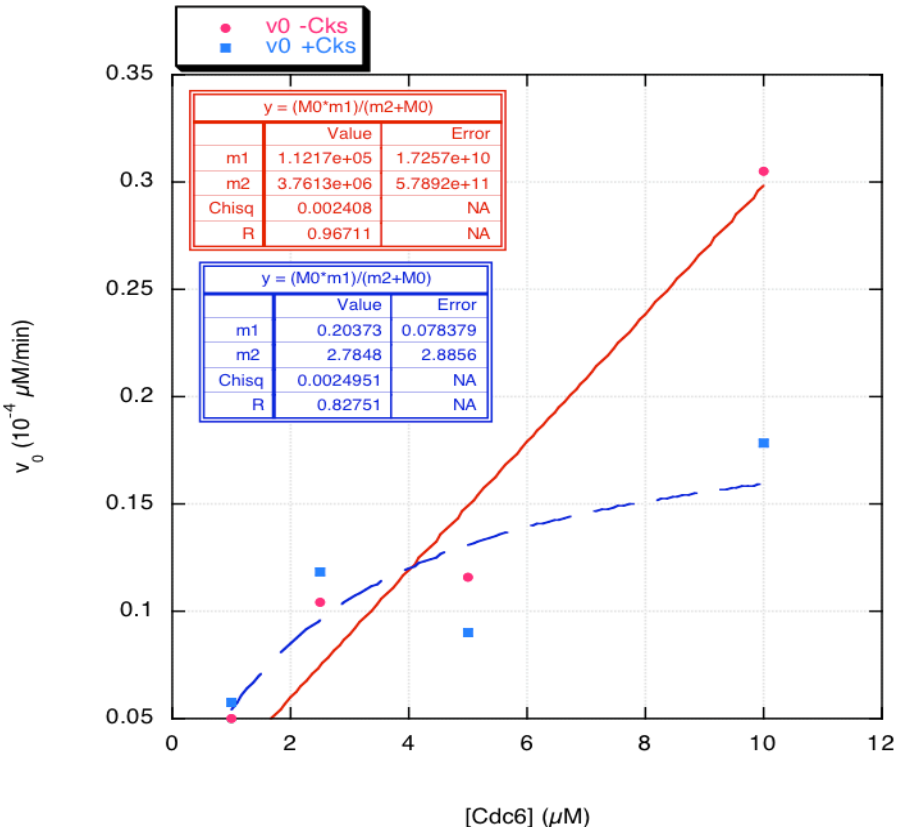


Figure 2.9. Experimental Determination of Kinetic Parameters of Cdc6 Phosphorylation by Clb2/Cdk1 ± Cks1. Initial velocities were plotted against Cdc6 concentrations and nonlinear regression of the Michaelis-Menten equation was used to determine K_m (m2) and V_{max} (m1).

of our experiment. If Cks1 concentrations are too low relative to the K_d of Cks1-Cdk1, association will not be favored. Conversely, there was also concern that at Cks1 concentrations in large excess of enzyme, free Cks1 might bind phosphoCdc6 and compete with Cdk-bound Cks1 for substrate binding, thereby inhibiting Cks1-dependent phosphorylation. In light of these concerns, a Cks1 titration was performed, keeping enzyme and substrate concentrations constant but varying Cks1 concentration from 0 to 5 μM (Figure 2.10). Initial velocity was

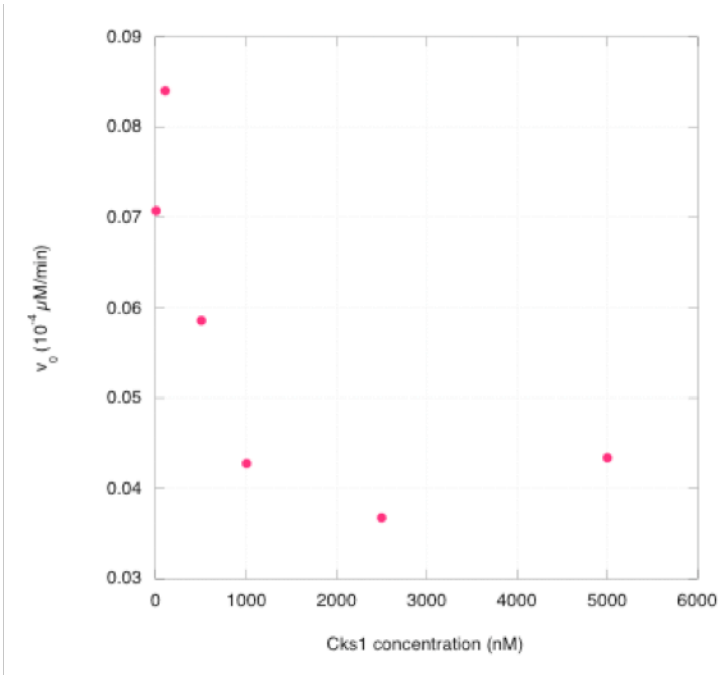


Figure 2.10. Initial Velocity is Minimally and Unpredictably Affected by Cks1 Concentration. Cdc6 was phosphorylated by Clb2/Cdk1 incubated with 0, 100 nM, 500 nM, 1 μM, 2.5 μM, or 5 μM Cks1 for four hours. Initial velocities were derived as described in Figure 2.8.

modestly higher (2-fold) at concentrations well below what would be expected to generate ternary complex, suggesting that the higher v_0 is not due to Cks1 but perhaps just inherent variability. High concentrations of Cks1 relative to Cdc6

concentration also failed to produce inhibitory effects. However, Cks1 concentration was not above the K_d for Cdc6 (10 μM) so formation of Cks1/Cdc6 complex would not be favored.

Given these results, we maintained our original assumption that using 1 μM Cks1 in a reaction with 100 nM Clb2/Cdk1 was appropriate. To my knowledge, the K_d of Cks1 for yeast Cdk1 has not been measured. Human Cks proteins bind Cdk2 with K_d = 50-80 nM reported in the literature, and K_d = 250 nM in my hands. We assumed that yeast Cks-Cdk K_d would be similar. Therefore we suspected that Clb2/Cdk1/Cks1 complex might be failing to form for reasons other than low affinity.

One possibility was that, despite the higher K_d of dimeric Cks1 compared to the Cdk1/Cks1 heterodimer, if the Cks1 added to Clb2/Cdk1 is entirely in the dimer form in solution and if the dimer has a slow off-rate, the ternary complex may not form in the allowed incubation time prior to the reaction. To test this hypothesis, Cks1-Cdk complex assembly was attempted using different Cks1 constructs that are known to be less prone to dimerization. Cks1(N1-C2) lacks C-terminal residues after 117, the so-called "glutamine tail." Truncation of Cks1 at residue 118 substantially destabilizes the dimer (K_d = 1 mM) (Bader et al. 2006). Alanine substitution at Pro93 also shifts the K_d of the dimer to ~90 mM (Bader et al. 2006). The Cks1 P93A construct used in this experiment was also truncated at residue 117. The effect of these two monomerizing mutations combined is not known. Wild-type Cks1, Cks1 (N1-C2), or Cks1 (N1-C2) P93A were incubated

with Clb2/Cdk1 prior to kinase assays performed at constant enzyme and Cdc6 NTD concentrations. No differences in v_0 were observed (Figure 2.11). We now know that the P93A substitution eliminates Cdk binding (Balog et al. 2011), although Cks1 P93A still binds phosphoproteins with wild-type affinity. Therefore the fact that the greatest (albeit modest) increase in initial rate was in the Cks1(1-2) P93A sample is especially meaningless.

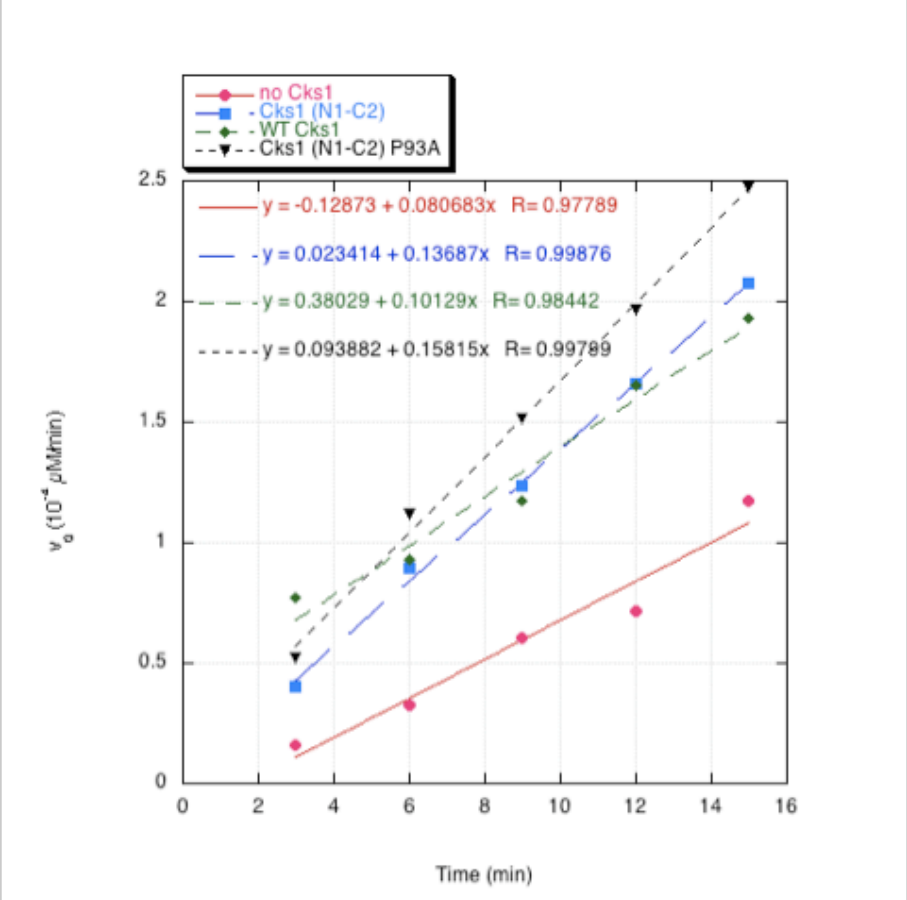


Figure 2.11. Using Different Cks1 Constructs Does Not Result in Dramatic Changes in Initial Rate. 1 μM Cdc6 was phosphorylated by 10 nM Clb2/Cdk1 in the presence of 1 μM Cks1 constructs. Initial rates (coefficients of x) were determined as described for previous experiments.

We then asked whether we might be in a substrate concentration range in which differences in v_0 were too small to detect reliably. At [S] >> K_m, differences in v_0 will be small. At [S] << K_m, v_0 will be very sensitive to substrate concentration and measurement inconsistency between samples could create substantial variability, obscuring Cks1-dependent effects. Given that the K_m for Clb2-Cdk1 toward a histone peptide substrate is ~50 µM, a range of Cdc6 concentrations was chosen below and above 50 µM to see at which

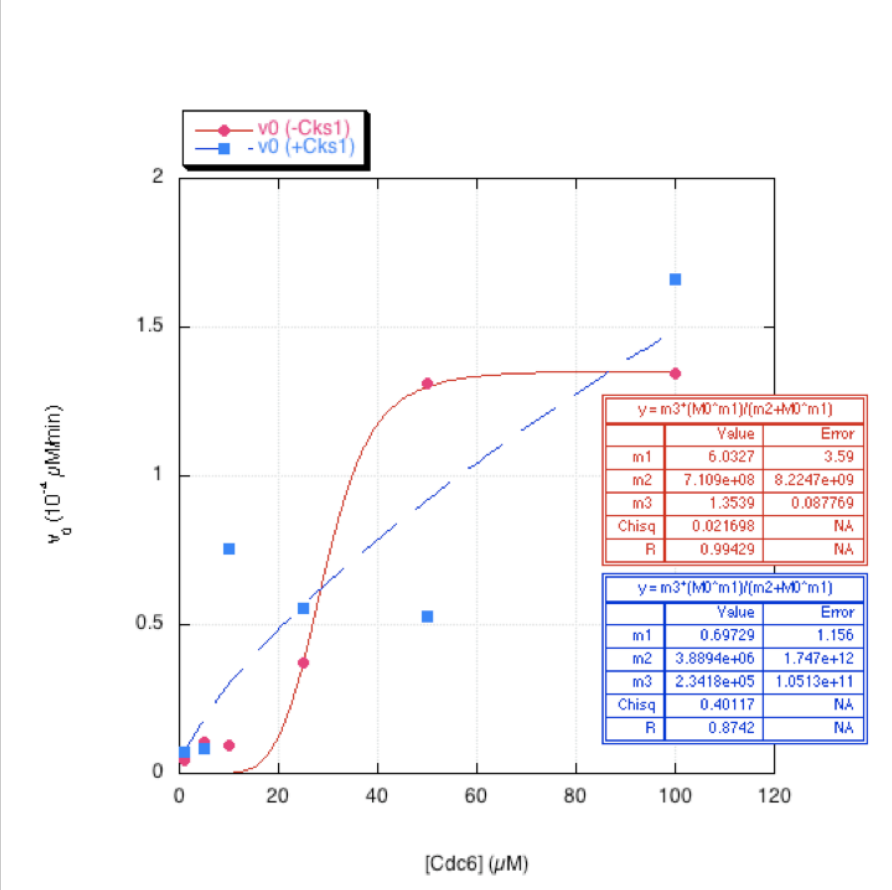


Figure 2.12. Initial Velocity of Cdc6 Phosphorylation by Clb2/Cdk ± Cks1 Over a Broad Substrate Range. Kinase reactions were performed at substrate concentrations of 1, 5, 10, 25, 50, and 100 µM. Initial rates were plotted as a function of substrate concentration and resulting curves were fit to the Hill equation.

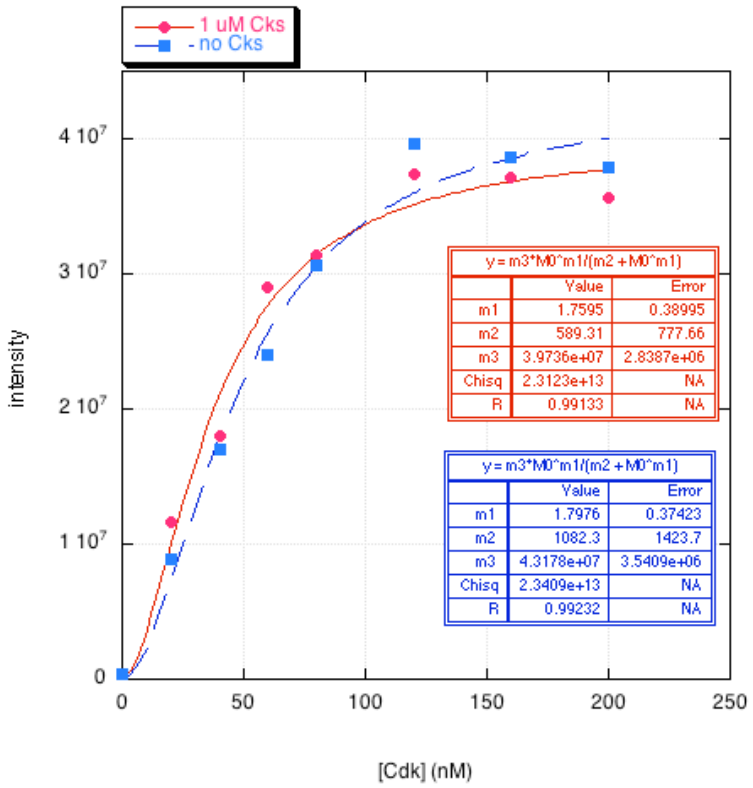


Figure 2.13. Ultrasensitivity in the Phosphorylation of Cdc6 NTD as a Function of Enzyme Activity ± Cks1. One micromolar Cdc6 NTD was phosphorylated by 0, 20, 40, 60, 80, 120, 160, or 200 nM Clb2/Cdk ± 1 μM Cks1. Overall phosphorylation was quantified and curves were fit to the Hill equation.

concentrations, if any, v_0 would diverge in the presence of 1 μM wild-type Cks1. The resulting curves are shown in Figure 2.12. Values for v_0 continued to increase by over 10-fold above v_0 determined for 10 μM Cdc6. Providing v_0 values closer to V_{max} should facilitate curve fitting. The greatest differences in v_0 ±Cks1 were still only approximately 2- to 3-fold, and in this particular case the +Cks1 reaction was slower. The -Cks1 reaction exhibited sigmoid kinetics when

fit to the Hill equation, while the Cks1+ reaction was too noisy to give meaningful parameters. Care was taken to ensure that these data were not mislabeled.

Given that some cooperativity was observed, we decided to look for a Cks1-dependent increase in cooperativity of Cdc6 phosphorylation using a simpler experiment. Phosphorylation of Cdc6 was plotted as a function of Clb2-Cdk1 concentration and the resulting curve was fit to the Hill equation to determine the Hill coefficient n_H (Figure 2.13). No differences in n_H were observed in the presence of Cks1.

Preassembly of Clb2/Cdk/Cks1 ternary complex had been avoided because it is generally considered bad practice to treat kinase samples differently before comparing them kinetically. However, as a last resort, we decided to preassemble Clb2/Cdk1/Cks1 using size exclusion chromatography. Purified, activated Clb2/Cdk1 was either directly purified over size exclusion or first incubated with an excess of pure wild-type Cks1 (Figure 2.14). The ternary

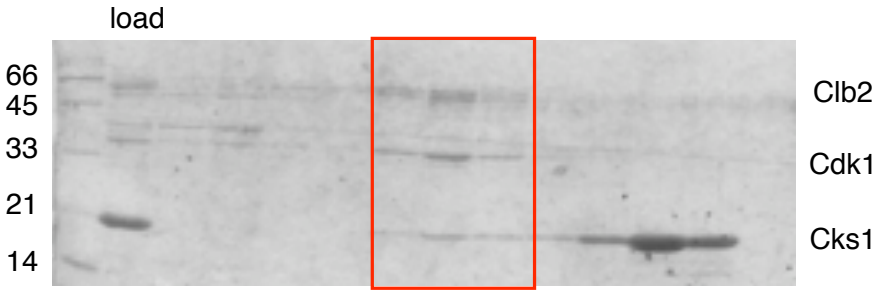


Figure 2.14. Assembly of Clb2/Cdk1/Cks1 Ternary Complex by Size Exclusion Chromatography. Clb2/Cdk1 was tandem purified by affinity chromatography. Following tag removal and activation by CAK, Clb2/Cdk1 was incubated with excess Cks1, concentrated, and run on a Superdex 75 column. Fractions considered ternary complex are boxed in red; excess unbound Cks1 can be seen in later fractions

complex was judged to be stoichiometric based on its appearance and on the presence of excess free Cks1. Clb2/Cdk1 or Clb2/Cdk1/Cks1 was incubated with 1 μM Swe1 NTD (residues 1-425), Swe1 NTD-N (residues 1-212), Sic1 (residues 1-100), or Cdc6 NTD (Figure 2.15). In each of these samples, phosphorylation by

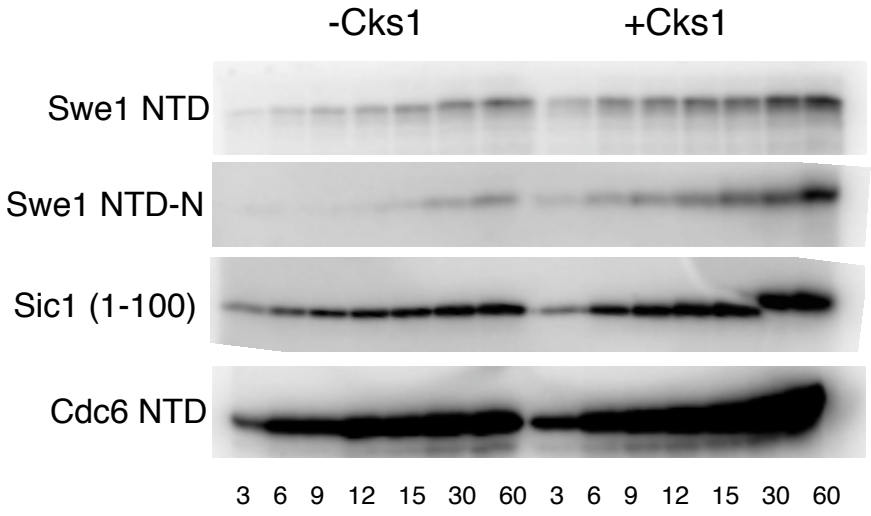


Figure 2.15. Phosphorylation of Multiply Phosphorylated Cdk Substrate Constructs by Pre-Assembled Clb2/Cdk1(/Cks1) Kinase. Time course kinase assays were performed as in Figure 2.7 except for the use of pre-assembled kinase complexes.

kinase assembled with Cks1 was both more rapid and to a greater extent in the 60-minute reaction than kinase without Cks1 (Figure 2.16). This was the first time that dramatic and reproducible (across substrates, at least) Cks1-dependent differences consistent with our hypothesis were observed. However, these results should be interpreted with caution. Importantly, there was no control for intrinsic enzyme activity, for example a singly phosphorylatable substrate such as Cdc6 Triple Mut. Without such a control, there is no way to determine

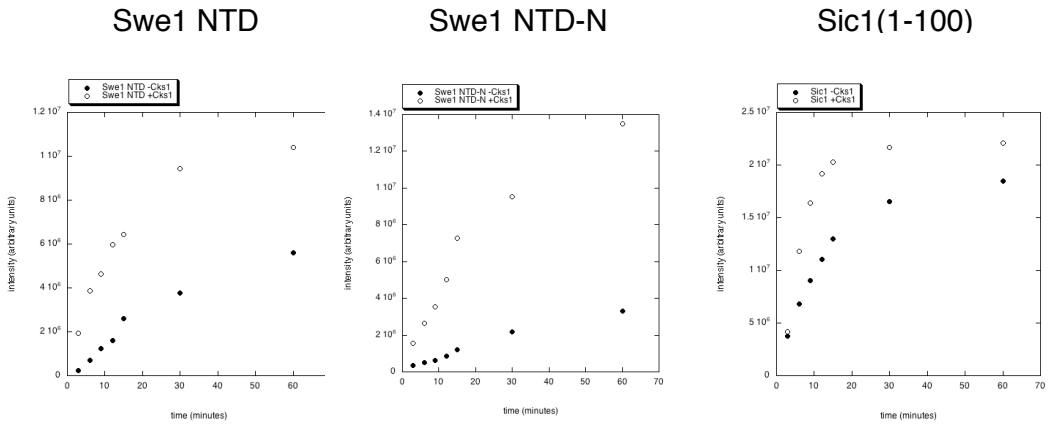


Figure 2.16. Quantitative Analysis of the Results in Figure 2.15. Cdc6 NTD phosphorylation was too overexposed for accurate quantitation.

whether the Clb2/Cdk1/Cks1 preparation is simply more active than the Clb2/Cdk1 preparation, despite care taken to prepare both samples side by side. Also, comparison of one substrate to another should be avoided. Cdc6 NTD may appear in this experiment to be the preferred substrate, but Swe1 NTD is less stable in solution and may have been less accessible for phosphorylation.

This section concludes with the results of a mass spectrometry experiment comparing the phosphorylation of Swe1 NTD by Clb2/Cdk1 in the presence and absence of Cks1. We hypothesized that Cks1 would affect the extent of phosphorylation of Swe1, and that at least some of Swe1's documented hyperphosphorylation was Cks1-dependent. Duplicate mass spectrometry experiments were performed to obtain site-specific phosphorylation information. His_6-Swe1 phosphorylated by pre-assembled Clb2/Cdk or Clb2/Cdk/Cks1 was trypsin-digested for either 3 hours (experiment 1) or overnight (experiment 2) at 37°C. After proteolysis was quenched with PMSF, reactions

were combined with an equal volume of 0.1% formic acid in water and processed with the help of Kyle Brown in the UCSC mass spectrometry facility. The best sequence coverage (~70%) was obtained in Experiment 2. In this experiment, all eight Cdk consensus sites that were phosphorylated in Harvey et al. (2005) were also phosphorylated in both the presence and absence of Cks1 (Table 2.1.). These experiments were intended to be initial tests of feasibility; analysis of nonconsensus phosphorylation was not performed.

Consensus Site		Phosphopeptide
-Cks1	+Cks1	
T45		K.QAGEDESDDFAIGGST*PTNK.L
S111		K.RWS*PFHENES*VTTPITK.R
T121		R.WSPFHENESVTT*PITK.R
S133		K.TNS*PISLK.Q
T196		R.IPET*PVKK.S
T196, S201		R.IPET*PVKKS*PLVEGR.D
S263		K.ALPSIHVPTIDSS*PLSEAK.Y
	S284*	R.HNNQTNILS*PTNSLVTNSSPQTLHSNK.F
S294*	S284, S294	R.HNNQTNILS*PTNSLVTNS*S*PQTLHS*NKFKK.I
	T373	R.GRYDNDTDEEIST*PTR.R
S418*		R.PLSLSSAIVTNTTSAETHSISSTDSS*PLNSK.R

Table 2.1. Swe1 Phosphorylation Sites Determined by Mass Spectrometry.
*S284, S294, and S418 are consensus Cdk sites that were only detected *in vivo* in Harvey et al. (2005), while the other sites were detected both *in vivo* and *in vitro.*

2.3 Discussion

It is not trivial to determine what various potential effects of Cks1 on multiphosphorylation by Cdk might look like as measured by our experimental design. Here I will attempt to depict those different effects mathematically and visually and perhaps explain why no measurable effects of Cks1 were observed in any of our experiments despite recent evidence supporting a role for Cks1 in processive phosphorylation of Sic1.

2.3.1. Experimental Design

There were a number of problems with our experimental design. Our prediction was that *specific* phosphorylation events were cooperative, not *all* phosphorylation. Necessarily, the first phosphorylation event on any Cdc6 molecule must occur independent of priming phosphorylation. Assuming that there is no intrinsic cooperativity to the system independent of Cks1-phosphoT7 association, every first phosphorylation event will be non-cooperative. If we assume that phosphoT7 is the only site capable of binding Cks1, and we also assume that the first phosphorylation event is equally likely to occur at any of the four sites in Cdc6, then there is only a one in four chance that the first phosphorylation event is priming. If we assume that after Cdc6 has been phosphorylated at the priming site, all other sites are phosphorylated in a cooperative manner, then 25% of Cdc6 proteins will possess three cooperatively-added phosphates (Figure 2.17). The remaining 75% has a one-in-three chance

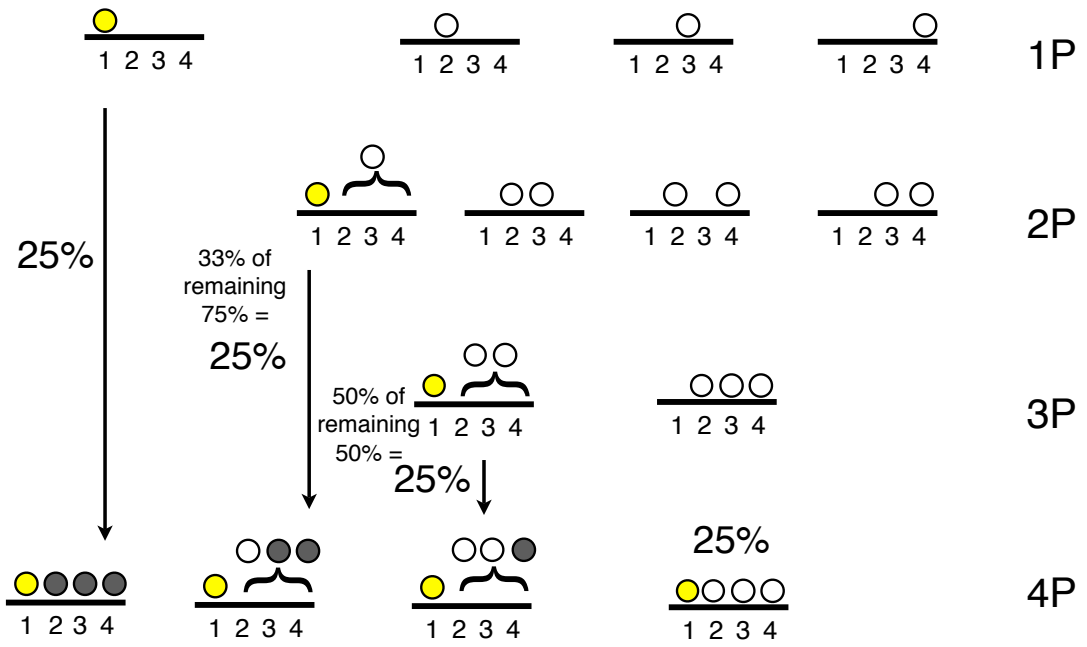


Figure 2.17. A Model of the Effects of Cks1 on Phosphorylation. This model assumes random phosphorylation site preference but that once the "special site" (yellow) is phosphorylated, all subsequent phosphorylation events will be Cks1-influenced. This model also assumes that no phosphorylation site is strictly Cks1-dependent. Grey sites represent Cks1-influenced phosphorylation events and white sites are phosphorylation events occurring with normal kinetics. Brackets indicate any position under the bracket may be occupied by the sites above the bracket.

of its next phosphorylation event occurring at the priming site. This means that 75% * 33% = 25% of Cdc6 proteins will possess two cooperatively-added phosphates. The remaining 50% of Cdc6 has a 50% chance of its third phosphorylation event occurring at the priming site, resulting in 50% * 50% = 25% of Cdc6 receiving their fourth and final phosphate in a cooperative manner. The remaining 25% of Cdc6 will receive all four phosphates without any cooperativity due to priming because the priming site is phosphorylated last. Figure 2.17 demonstrates that under these assumptions, only six of every 16, or

37.5%, of phosphates observed will be added in a way that contributes to $n_H > 1$. The same logic applies to the relative contribution of Cks1-dependent lower K_m to the apparent K_m of the reaction in the presence of Cks1. The relatively small contribution of Cks1-driven events to these apparent parameters means that differences ±Cks1 would have had to have been large in order to have been consistently observed.

Our cooperativity experiment (Figure 2.13) was modeled on previous work from the Ferrell lab measuring the Hill coefficient of Wee1 phosphorylation (Kim and Ferrell 2007). In Figure 1c of Kim & Ferrell (2007), phosphorylation of Wee1 T150 was measured as a function of CycB/Cdk1 concentration using a phosphospecific antibody. In the future, it would be advantageous to use an experimental technique that reports the precise output expected to be cooperatively generated by the stimulus. For example, a phosphospecific antibody could report the phosphostate of a particular site as an observable. Alternatively, the Loog lab's PhosTag gel technique allows for the separation of phosphoforms, so one could determine which phosphoforms appear with sigmoidal versus hyperbolic kinetics.

It is worth examining our choice of enzyme and substrate combination, allowing for the possibility that we simply did not find a good system with which to test our hypothesis. Even though Cdc6 is a preferred Clb5/Cdk1 substrate, it is probably also a substrate for Clb2/Cdk1, as its CDK sites have been found to be required for its proteolysis in mitosis (Loog and Morgan 2005; Perkins, Drury,

and Diffley 2001). When Clb2/Cdk1 phosphorylates Cdc6, it does so independently of the hydrophobic patch on Clb2 (Kõivomägi, Valk, Venta, Iofik, Lepiku, Morgan, et al. 2011). Cdc6 also efficiently inhibits Clb2/Cdk1, but not other cyclin-Cdk1s, in a phosphorylation-dependent manner. This presents a significant practical problem for deriving a Hill coefficient for the phosphorylation of Cdc6 (Ricard and Cornish-Bowden 1987). It is difficult to imagine how Cks1-phosphoCdc6 interaction could be sufficient to explain Clb2/Cdk inhibition, since Cks1 is also present in other cyclin-Cdk1 complexes that are not inhibited by phosphoCdc6. One possible explanation is site-specificity. If only Clb2/Cdk1 can phosphorylate a Cks1-binding site, then only Clb2/Cdk1 will produce inhibitory Cdc6. It is possible that Cdc6 inhibitory effects confounded our kinetic analysis. That is why comparison across multiple substrates will be essential. However, if Cks1 binding turns out to be a general mechanism of Cdk inhibition experiments to determine the kinetic parameters and mode of inhibition will be of interest. For example, we may ask to what extent inhibition is substrate competition versus competitive binding of a not-further-phosphorylatable substrate molecule. In this way, a story of Cks1 kinetics may be salvaged and combined with a story of binding specificity.

2.3.2. Processivity

Our experiments were not designed to measure processivity. In fact, we did not necessarily predict processive phosphorylation because most examples of ultrasensitive multisite phosphorylation kinetics are distributive (J. E. Ferrell

Jr. and Bhatt 1997; Nash et al. 2001). However, Cks1 clearly makes phosphorylation more processive in the case of Sic1. What does this mean for the role of Cks1 in the ultrasensitive degradation of Sic1? It is important to remember that only two distributive events need to occur to generate ultrasensitivity, as demonstrated in the dual phosphorylation of MAP kinases (J. E. Ferrell Jr. and Bhatt 1997). The multiphosphorylation of Sic1 is more processive in the presence of Cks1, but is also necessarily distributive because it is phosphorylated by two different cyclin-Cdk complexes in series (Kõivomägi, Valk, Venta, Iofik, Lepiku, Balog, et al. 2011). In the case of Sic1, processivity contributed by Cks1 makes Sic1 a better substrate for the second step of the phosphorylation cascade. It will be interesting to see if other multiply phosphorylated proteins combine processive and distributive phosphoregulation for specific regulatory purposes.

2.3.3. Ultrasensitivity and Cks1

My objective in this section is to discuss the ways that Cks1 might contribute to ultrasensitivity, so that future work may formally demonstrate the necessity of Cks1 to ultrasensitive responses generated by multisite phosphorylation of Cks1-directed substrates. For the sake of simplicity and relevance, when discussing the cooperativity of enzymatic modifications I will refer to a kinase/phosphatase system, although everything is generalizable for any reversible modification.

Most commonly the concept of cooperativity is associated with ligand binding and allosteric interactions such as those in hemoglobin. Cooperativity may also arise in covalent modification schemes such as phosphorylation. Goldbeter and Koshland first analyzed this phenomenon and the corresponding concept of "ultrasensitivity" (Goldbeter and Koshland 1984; Goldbeter and Koshland 1981). Goldbeter and Koshland describe three potential ways that an enzyme-mediated signal could increase in sensitivity beyond typical hyperbolic Michaelis-Menten kinetics.

First, conventional cooperative ultrasensitivity could arise if the enzyme is allosterically regulated. An enzyme possessing multiple substrate binding sites that interact to increase successive association constants for substrate exhibits this type of cooperativity (Ricard and Cornish-Bowden 1987). Since Clb2/Cdk1 has only one active site and does not form multimers to our knowledge, we can ignore allostery between catalytic subunits as a source of ultrasensitivity. Second, multistep ultrasensitivity could arise if a ligand/substrate acts at more than one step in the pathway from stimulus to observable, amplified response (for example, by participating in feed-forward or feedback signaling). Ultrasensitivity generated by distributive multisite phosphorylation is an example of multistep effects. The third type of ultrasensitivity described in the Goldbeter-Koshland model is zero-order ultrasensitivity. Zero-order ultrasensitivity can be generated if kinetics of at least one modification must operate in the zero-order range, meaning that the enzyme(s) are saturated with

substrate. It is only in this regime that small changes in the ratio of kinase to phosphatase can have abrupt effects on the overall phosphorylation state of the substrate. We have thus far ignored dephosphorylation entirely because we were attempting to reconstitute a system to observe the kinetic effects of Cks1 with as few components as possible. Importantly, only one enzyme of an opposing pair need be operating with zero-order kinetics to generate ultrasensitivity. However, the presence of zero-order effects cannot be experimentally determined without conditions that represent a steady-state system, requiring both kinase and phosphatase activity. Hypothetically, Cks1 could generate zero-order effects by increasing effective Swe1 concentration to Clb2/Cdk's zero-order range.

Additional sources of ultrasensitivity have been described. Competition between different substrates for access to an enzyme may generate ultrasensitivity (M. Chen et al. 1997). Nonprocessive multisite phosphorylation of a single substrate molecule is analogous to substrate competition if each phosphoacceptor site is considered "substrate." This type of intrinsic competition has been observed to generate ultrasensitivity in the case of Wee1; however, because these experiments were only performed in the presence of Cks1, it is not clear whether or to what extent Cks1 contributes to observed ultrasensitivity (Kim and Ferrell 2007). Regardless, Cks1 should not be strictly necessary for ultrasensitivity generated by intrinsic substrate competition if phosphorylation is at all distributive.

It has also been shown theoretically that if one or both opposing enzymes acquire affinity for their product(s), this creates ultrasensitive effects due to enzyme competition for substrate (Salazar and Höfer 2006). It has been proposed that ultrasensitivity of Swe1 phosphorylation could be due to zero-order effects or competition between Clb2/Cdk1 and $PP2A^{Cdc5}$. However, the observed ultrasensitivity in Swe1 phosphorylation can be fully accounted for using a multistep effect model (Harvey et al. 2011). Again, it is not clear to what extent Cks1 may participate in generating these effects, but Cks1 clearly could provide a mechanism for Clb2/Cdk1 to acquire affinity for Swe1.

2.4 Materials and Methods

2.4.1. Cyclin Expression and Purification

GST-Clb2 is expressed in *E. coli* from a pGEX vector with a TEV cleavage site. Expression is induced at OD_{600} = 0.6-1.0 overnight at 22°C using 1 mM IPTG. Cells are resuspended and lysed in lysis buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM PMSF) using two passes through a cell disruptor and lysates are clarified by centrifugation for 30 minutes at 17,000 rpm. Clarified lysates are applied to GS4B gravity column (usually 10-20 ml of resin for a 6-8L preparation of *E. coli*) equilibrated in wash buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM DTT). Column is washed extensively with wash buffer and GST-Clb2 is eluted using wash buffer + 10 mM glutathione, pH adjusted to 8.0. Expected yield for 6L is 60-200 mg.

2.4.2. Kinase Expression, Purification, and Activation

S. cerevisiae Cdk1 was expressed from either a pVev vector cloned by Seth Rubin at Sloan-Kettering or from a Bac-to-Bac virus generated by Will Finch using a pFastBac vector containing the Cdc28 gene. *Sf9* cells are infected at a density of 1.5 x 10^6 cells/ml with 10 ml of P2 viral stock per liter of culture. After 72 hours at 27°C, cells are harvested by centrifugation at 3500 rpm. Cell pellets may be stored at -20°C or -80°C for long-term storage. Thawed or fresh cell pellets are resuspended in 100 ml of 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM PMSF, 1 µM leupeptin, and 1 µM pepstatin per liter of cells. Resuspended cells are lysed using two passes through the cell disruptor. Lysates are clarified by spinning for 2x30 min at 18,000 rpm, switching to fresh centrifugation tubes in between spins. Extra centrifugation may be necessary if lysates are still cloudy. Clarified lysates can then be applied to either an equilibrated Ni^{2+} column or an equilibrated GST-Clb2 column for complex assembly. Eight milliliters of GS4B resin is equilibrated in lysis buffer. At least 20 mg of GST-Clb2 is applied to the GS4B resin and allowed to flow through. Care should be taken that GST-Clb2 has been purified away from glutathione before this step. Cdk1 supernatant is then applied to the resin and allowed to flow through at a rate not to exceed 1 drop/ second. The resin is washed with wash buffer (25 mM Tris-HCl pH 8.0, 200 mM NaCl) and GST-Clb2/His_6-Cdk1 is eluted with wash buffer + 10 mM glutathione pH = 8. GS4B-purified kinase is then applied to 5 ml of Ni^{2+} resin equilibrated in 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM imidazole. Resin is washed with

the same buffer as equilibration and eluted 5 ml at a time with equilibration buffer + 400 mM imidazole. Fusion tag cleavage and CAK activation may be performed simultaneously at 4°C overnight with 10% w/w CAK, 1 mM ATP, 10 mM $MgCl_2$, and 1-2% w/w TEV protease. Clb2/Cdk1 is then cleaned up over GS4B resin to remove GST tag. If further purification is needed, Clb2/Cdk1 is purified on mono Q resin at pH 8 or, for greater purity, by size exclusion chromatography on a Superdex 200 column in 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM DTT. If assembling complex with Cks1, 3-fold molar excess Cks1 is added prior to size exclusion. Cells and lysate should be kept cold throughout purification but avoid freezing in between purification steps if possible. The best His_6-Cdk1 expression achieved so far is ~6mg/L. After activation and purification, kinase was concentrated to a point where a small volume could be added to a 50 µl kinase reaction for a final concentration of 10-50 nM. Clb2/Cdk1 is approximately 80 kDa, so concentrations between 0.1-0.25 mg/ml were suitable.

2.4.3. Radioassays

To ensure consistency between samples, a 5X mastermix was made that resulted in final concentrations of 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT, 0.2 mM ATP, 10 mM $MgCl_2$, and 1-10 µCi of ^{32}P-ATP per reaction. Typical substrate concentrations were 1-10 µM and kinase concentrations were 10-50 nM. Kinase was added last and time of addition was defined as t = 0. At each time point, 10 µl of kinase reaction was removed and quenched in 5 µl protein loading

buffer. Five µl of quenched sample was then resolved by gel electrophoresis on a 17.5**%** polyacrylamide gel made with the Kellogg lab's gel recipes until excess ^{32}P-ATP was run off the gel, which usually corresponded to about 5 minutes after the dye front ran off at 200 V. Gels stored in destain (5% methanol, 7% acetic acid) for at least 20 minutes prior to drying seemed to survive the drying process better. Dried gels were exposed overnight to a phosphor screen and screen images were scanned using a Typhoon scanner**.** Rectangles of identical size were drawn around each band were background corrected using a single rectangle of the same size in blank space on the image. Volumes were quantified using ImageQuant. All original processed images are saved as 'date/assay processed'. Raw data were fit to the integrated first-order rate equation in the form of the exponential recovery equation, which in Kaleidagraph is written "y = m1*(1-exp(m2*M0)). Typical initial variables for curve fitting were m1 = 1,000,000, m2 = -10. To normalize, the variable m1, which corresponds to y as x (M0) goes to infinity, is set to total substrate concentration (protein concentration multiplied by number of phosphorylation sites per substrate molecule).

2.4.4. Mass Spectrometry

His_6-Swe1 NTD (62.5 µg in Experiment 1, 20 µg in Experiment 2) was phosphorylated by Clb2/Cdk1 or Clb2/Cdk1/Cks1 that had been preassembled by size exclusion chromatography (the same enzyme stocks used in Figures 2.14-2.16) in a total volume of 50 µl containing 1 mM ATP, 10 mM $MgCl_2$, 25 mM Tris-HCl pH 8.0, 100 mM NaCl, and 5 mM DTT. Kinase reactions were incubated

for one hour at room temperature and quenched with 5 µl 0.5M EDTA pH 8.0. Three milligrams of trypsin were dissolved in one milliliter of 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT. A total of 1 µg trypsin and 1 mM $CaCl_2$ was added to quenched kinase reactions and digests were incubated 3 hours (Experiment 1) or overnight (Experiment 2) at 37°C. Trypsin digests were terminated with 1 mM PMSF. Mass spectra were obtained using a Thermo Finnigan LC/MS/MS (LTQ) and processed using a SEQUEST algorithm.

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Chapter 3: A Monomer Mutant Cks Protein Reveals Multiple Roles for a Conserved Hinge-Region Proline

3.1. Abstract

Cks (cyclin-dependent kinase subunit) proteins are essential eukaryotic cell cycle regulatory proteins that physically associate with cyclin-dependent kinases (Cdks) to modulate their activity. Cks proteins have also been studied for their ability to form domain-swapped dimers by exchanging β-strands. Domain swapping is mediated by a conserved β-hinge region containing two proline residues. Previous structural studies indicate that Cks in its dimer form is unable to bind Cdk, suggesting that the monomer–dimer equilibrium of Cks may have an effect on Cks-mediated Cdk regulation. We present the crystal structure of a proline-to-alanine mutant Saccharomyces cerevisiae Cks protein (Cks1 P93A) that preferentially adopts the monomer conformation but surprisingly fails to bind Cdk. Comparison of the Cks1 P93A structure to that of other Cks proteins reveals that Pro93 is critical for stabilizing a multiple β-turn structure in the hinge region that properly positions an essential Cdk-binding residue. Additionally, we find that these β-turn formations, conserved in Cks homologs, have implications for the mechanism and preferentiality of strand exchange. Together, our observations suggest that the conservation of Cks hinge-region prolines reflects their functions in forming a Cdk binding interface and that the ability of these prolines to control partitioning between monomer and dimer is a consequence of the β-turn networks within the hinge.

3.2. Introduction

The eukaryotic cell cycle is coordinated by cyclin-dependent kinases (Cdks), whose activities are tightly regulated by a variety of biochemical mechanisms (Morgan 1995; Pavletich 1999). One of the least understood mechanisms of Cdk regulation is association with Cks proteins. Cks proteins are small (9-18 kDa), conserved, cell cycle regulatory proteins that physically interact with Cdks (Patra and Dunphy 1996; Bourne et al. 1996; Hadwiger et al. 1989; Pines 1996; Richardson et al. 1990). The importance of Cks proteins is evident from observations that their loss causes severe cell cycle defects and lethality in model organisms and that Cks is upregulated in a number of human cancers (Hayles, Aves, and Nurse 1986; Inui et al. 2003; Lan et al. 2008; Shapira et al. 2004; Tang and Reed 1993; Westbrook et al. 2009). Structural and biochemical evidence suggests that Cks targets Cdks to phosphoprotein substrates (Bourne et al. 1996; Patra et al. 1999); however, the precise functions of Cks proteins and their importance in particular cell cycle events are not well characterized.

Outside of their role in cell cycle regulation, Cks proteins have been extensively studied for their conserved ability to form domain-swapped dimers (Bader et al. 2006; Rousseau et al. 2001; Schymkowitz, Rousseau, and Itzhaki 2000; Seeliger, Breward, and Itzhaki 2003; Seeliger et al. 2002; Seeliger et al. 2005; Schymkowitz et al. 2001), a distinctive type of protein-protein interaction (Bennett, Schlunegger, and Eisenberg 1995). Cks proteins dimerize by

exchanging β-strands via extension of a conserved hinge region; exchange occurs via a denatured intermediate state. Both the *Saccharomyces pombe* Cks homolog p13suc1 (suc1) and *Saccharomyces cerevisiae* Cks1 exist *in vitro* as both monomer and dimer, with dimerization constants ~2mM and ~0.4 mM respectively (Bader et al. 2006; Rousseau et al. 2001). The human Cks homologs CksHs1 and CksHs2 also have some propensity for dimerization, albeit weaker than the yeast orthologs (Seeliger et al. 2002; Seeliger et al. 2005). Studies of suc1 and CksHs1 suggest that exchange between monomer and dimer occurs through a partially unfolded intermediate state (Rousseau et al. 2001; Seeliger et al. 2002).

The crystal structure of human Cdk2 in complex with CksHs1 shows that CksHs1 binds Cdk2 as a monomer (Bourne et al. 1996). Molecular modeling predicts that Cks in its strand-exchanged dimer form cannot bind Cdk for a number of reasons, including steric hindrance between Cdk and the second subunit of a Cks dimer and the absence of important contacts between Cdk and the hinge region of Cks. Together, the conserved nature of dimer formation in Cks proteins and the fact that only monomeric Cks can bind Cdk suggest that the monomer-dimer equilibrium may affect Cks-mediated Cdk regulation *in vivo*. The dissociation constants for the monomer-dimer equilibria of individual hinge region mutants have been characterized in Cks1 and in suc1 (Bader et al. 2006; Rousseau et al. 2001). These studies identified two hinge region proline residues, Pro93 and Pro95 (*S. cerevisiae* numbering), which most significantly

affect swapping between monomer and dimer. Interestingly, mutation of Pro93 favors the monomer while mutation of Pro95 drives dimer formation. It has been proposed that the conformational restrictions of these prolines tune the strain in the hinge region to favor or disfavor oligomerization (Rousseau et al. 2001). Although measuring the K_d of human Cks protein dimers has been technically challenging, the K_d of CksHs1 dimer has been estimated to be in the 10 mM range, and mutation of Pro62 (analogous to Cks1 Pro93) was reported to increase the K_d by at least an order of magnitude (Seeliger et al. 2005). Previous work has also shown that mutation of multiple CksHs1 residues in or near the hinge region (Tyr57, Met58, His60, and Glu63) in combination or mutation of Glu63 alone disrupts Cdk binding (Bourne et al. 1996). However, because the ability of Cks hinge region proline mutants to bind Cdk has not been assessed, and because some hinge region residues are involved in direct contacts with Cdk2, it is difficult to deconvolute the contribution of hinge residues to dimerization from their roles in the Cdk binding interface.

In order to characterize further a role for domain swapping in Cks-Cdk regulation, we test and describe here the effects of shifting the monomer-dimer equilibrium of Cks on Cdk binding. We present the novel structure of a budding yeast Cks mutant (Cks1 P93A) that preferentially exists as a monomer but fails to bind Cdk. Our structure demonstrates the critical role of this proline residue in Cdk binding and reveals the importance of hinge region residues for domain-swapping and Cks function.

3.3. Results

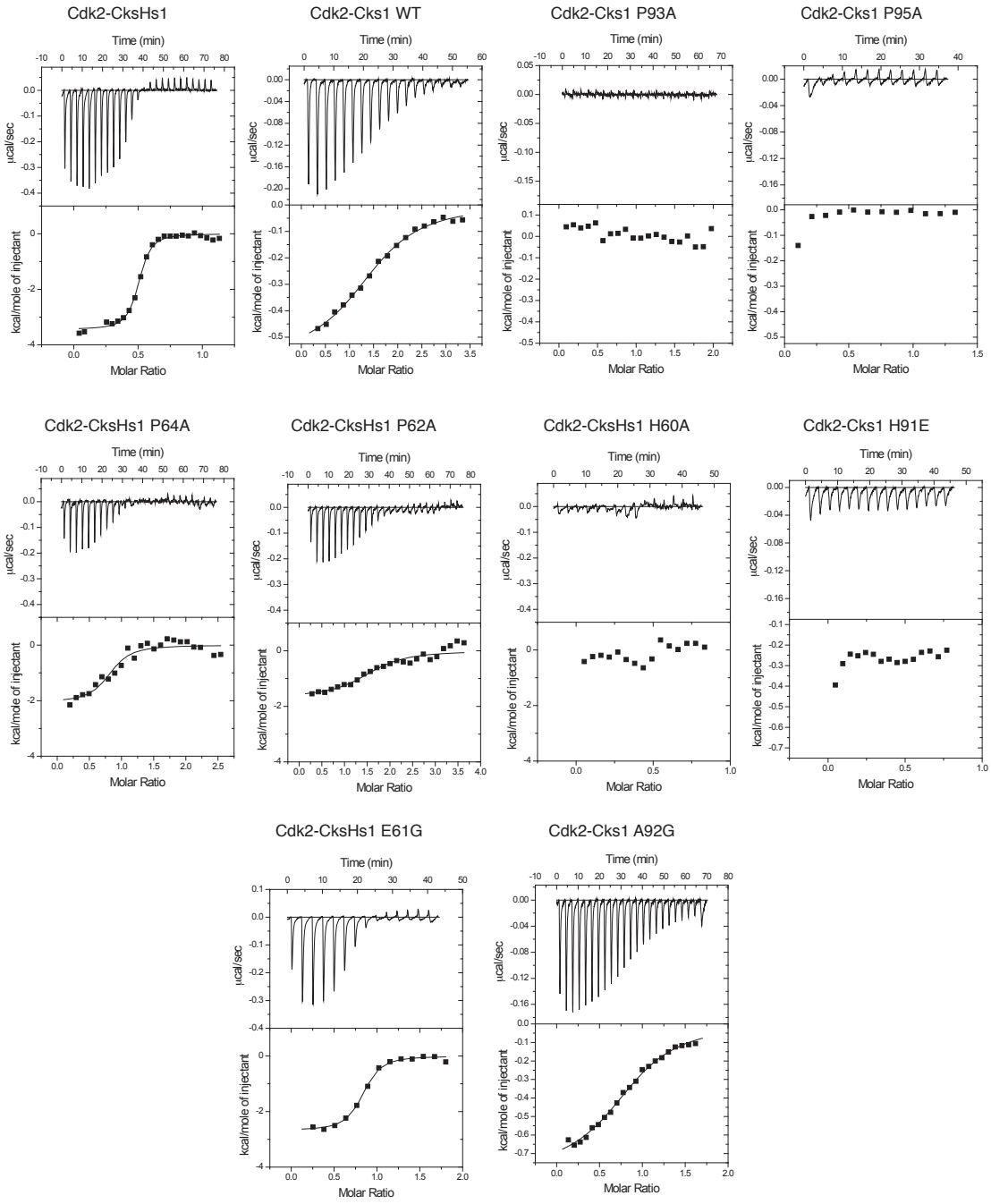
3.3.1. Mutation of Cks Hinge-Region Prolines Inhibits Cdk Binding

We hypothesized that the monomer-dimer equilibrium of Cks proteins regulates Cks association with Cdks and accordingly that the propensity for dimer formation is negatively correlated with the ability to bind Cdk. To test this hypothesis, we carried out a series of Isothermal Titration Calorimetry (ITC) experiments to quantify the effects of mutations that stabilize either monomer or dimer Cks on Cdk binding. In order to obtain sufficient quantities of Cdk for ITC, we chose to express and purify human Cdk2 from Sf9 insect cells. The Cks-Cdk binding interface is highly conserved, allowing wild-type yeast Cks1 to form a stable complex with human Cdk2 and CksHs1 and CksHs2 to functionally compensate for Cks1 in yeast (Richardson et al. 1990). The K_d of wild-type Cks1 for Cdk2 was determined to be 16 ± 1 μM (Figure 3.1). This value is approximately 64-fold weaker than our measured K_d of CksHs1 for Cdk2 (Figure 3.1, Table 3.1).

S. cerevisiae		***H. sapiens***	
Cks Protein	Cdk2 Kd (μM)	Cks Protein	Cdk2 Kd (μM)
WT	16 ± 1	WT	0.25 ± 0.04
H91E	NB	H60A	NB
A92G	14 ± 1	E61G	0.26 ± 0.01
P93A	NB	P62A	1.60 0.95
P95A	NB	P64A	0.63 ± 0.12

Table 3.1. Dissociation Constants for Cdk2-Cks1 and Cdk2-CksHs1. NB = no detectable binding.

Figure 3.1. **Representative Isothermal Titration Calorimetry (ITC) Data for Cks-Cdk2 Binding Experiments.** Concentrations and binding constants are described in Table 3.1. and Materials and Methods.


Cdk2-CksHs1
Cdk2-Cks1 WT
Cdk2-Cks1 P93A
Cdk2-Cks1 P95A
Cdk2-CksHs1 P64A
Cdk2-CksHs1 P62A
Cdk2-CksHs1 H60A
Cdk2-Cks1 H91E
Cdk2-CksHs1 E61G
Cdk2-Cks1 A92G
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

We next expressed and purified a series of yeast and human Cks mutants containing single amino acid substitutions in the hinge region. The effects of these substitutions on monomer-dimer equilibrium were evaluated using size-exclusion chromatography performed at concentrations comparable to those used in calorimetry experiments and were consistent with previously reported results for Cks1 proline-to-alanine mutants (Figure 3.2) (Bader et al. 2006). We

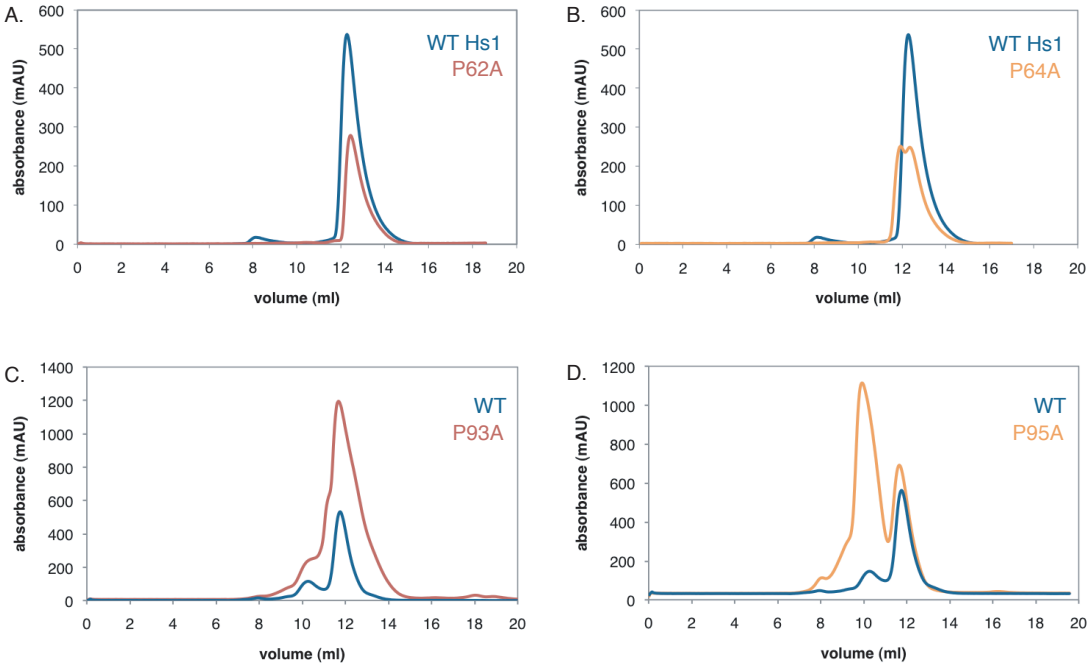


Figure 3.2. Size Exclusion Chromatograms of Cks Proteins. (a) CksHs1 (dark blue, 0.3 mM)) and CksHs1 P62A (red, 0.14 mM) (b) CksHs1 (dark blue, 0.3mM) and CksHs1 P64A (orange, 0.15 mM). CksHs1 elutes as a single peak while CksHs1 P64A elutes as a double peak. The elution profile of CksHs1 P64A is similar to previous size exclusion chromatography results for the CksHs1 E63P mutant, which has higher domain swapping propensity than wild-type CksHs1 (Seeliger et al. 2002). Based on this similarity and the effects of alanine substitution at this position in homologs, we predict that CksHs1 P64A also forms domain-swapped dimers in solution. (c) Cks1 (dark blue, 0.26 mM) and Cks1 P93A (red, 0.9 mM). (d) Cks1 (dark blue, 0.26 mM) and Cks1 P95A (orange, 0.84 mM).

predicted that stabilizing dimeric Cks1 (P95A mutation) would weaken binding of Cks1 to Cdk2 and that stabilizing monomeric Cks1 (P93A mutation) would strengthen binding to Cdk. We found that Cks1 P95A did in fact fail to bind Cdk2, with a K_d too weak to measure. Surprisingly, Cks1 P93A also failed to bind Cdk2, despite the preference of the P93A mutant for the monomer conformation.

We also made the analogous mutations to CksHs1 and measured their binding affinities to Cdk2. Considering the high structural homology between Cks1 and CksHs1 (rmsd =1.20 Å for 61 C atoms), it has been predicted that mutations to CksHs1 Pro62 and Pro64, the conserved hinge region prolines corresponding to Cks1 Pro93 and Pro95, would also exhibit conserved effects in stabilizing and destabilizing the monomer form, respectively (Seeliger, Breward, and Itzhaki 2003; Seeliger et al. 2002). Indeed, we observed that CksHs1 behaves as a monomer during size exclusion chromatography while CksHs1 P64A elutes as a double peak, presumably corresponding to both monomer and dimer forms (Figure 3.2d). We found that CksHs1 P64A binding to Cdk2 was reduced 2.5-fold compared to wild-type, consistent with a decrease in monomer stability. In agreement with our results for Cks1 P93A, CksHs1 P62A exhibited a 6-fold reduction in binding to Cdk2. CksHs2 also binds Cdk2 with a similar affinity as CksHs1 (Watson et al. 1996), however we could not test binding of CksHs2 hinge-region mutant proteins due to their instability upon recombinant expression.

The observation that alanine substitution at Pro62/Pro93 results in a loss of Cdk binding is noteworthy given that this Pro62 does not make a direct contact with Cdk2 and that alanine substitution at this position shifts the strand-exchange equilibrium toward the monomer conformation (Bourne et al. 1996; Rousseau et al. 2001). If the monomer fold is indeed adopted under specific biological conditions to promote Cks-Cdk association, the monomeric Cks1 that occurs *in vivo* must be structurally distinct from the P93A monomer. To obtain a structural perspective on the effects of this substitution, we crystallized and solved the structure of monomeric Cks1 P93A.

3.3.2. Overall Structure of Cks1 P93A

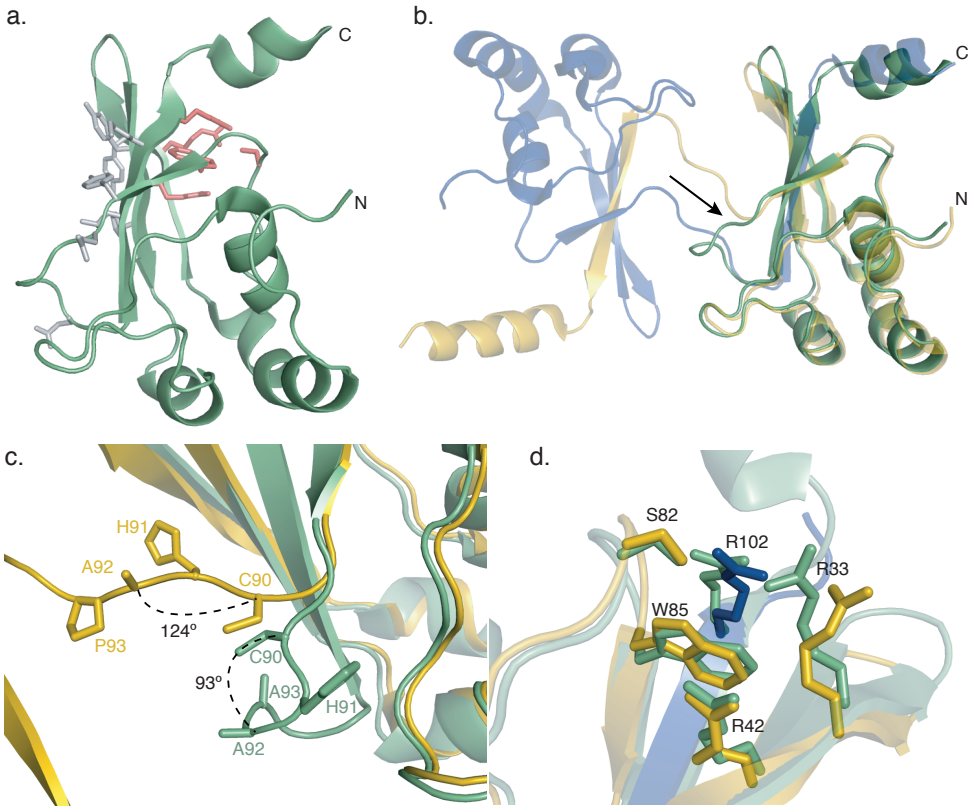
We obtained crystals of Cks1 P93A using a mutant version of a truncated *S. cerevisiae* Cks1 (residues 1-117). The structure was determined by molecular replacement using a search model created from two molecules of dimeric Cks1 lacking the hinge loop (residues 6-88, 94-105). The final model was refined to 2.6 Å (see Table 3.2 for diffraction data and refinement statistics).

Cks1 P93A crystallized as a monomer (Figure 3.3a). The overall fold of Cks1 P93A is essentially identical to wild-type dimeric Cks1 (rmsd = 0.53 Å for C of residues 7-89, Figure 3.3b) with the exception of the hinge region between the third and fourth β-strands, which is responsible for the strand exchange (Figure 3.3b, arrow). The β-4 strand of the monomer aligns with the swapped strand of the adjacent molecule in the dimer (rmsd = 1.43Å for C of residues 95-111). The greatest differences between wild-type Cks1 and Cks1 P93A lie in the flexible

Table 3.2. Summary of Crystallographic Analysis. *Values in parentheses are for the outer resolution shell.

Data Collection	
Beamline	ALS-5.0.1
Space Group	$P4_32_12$
Cell Dimensions (Å)	a = b = 89.5, c = 93.2
Resolution (Å)	64.55-2.59 (2.64-2.60)*
R_{sym}	8.1 (41.0)
I/(I)	31.2 (5.4)
Completeness (%)	100 (100)
Redundancy	9.4 (9.3)
Refinement	
Resolution (Å)	64.55 - 2.59
Total reflections	249329
Unique reflections	12176
R_{work}/ R_{free}	21.1/27.3
Total protein atoms	1829
Water molecules	42
Rmsd	
Bond lengths (Å)	0.013
Bond angles (°)	1.53
B factors	
Protein	25.2
Water	26.7
Ramachandran Analysis (%)	
Preferred	97.09
Allowed	1.94
Outliers	0.97

Figure 3.3. Overall Structural Comparison of Monomer and Dimer Cks1. (a) Structure of Cks1 P93A, which crystallizes as a monomer. Conserved protein-protein interaction surfaces are shown in stick model. Cdk-binding residues, as predicted from the structure of CksHs1 bound to Cdk2, are colored gray; residues comprising the anion-binding pocket are colored pink. (b) Cks1 P93A (green) aligned with wild-type dimeric Cks1 (PDB ID: **1QB3**, shown in yellow and blue). The arrow denotes the hinge region. (c) Close-up of the hinge region of Cks1 P93A (green) aligned with wild-type dimeric Cks1 (yellow). C^{α} atoms of Cys90, His91, and Ala92 were used to calculate the overall angle of the hinge; these residues as well as the proline that underwent mutation to alanine (Pro/Ala93) are shown in sticks. (d) Close-up of the anion-binding pocket of Cks1 P93A (green) compared with the anion-binding pocket of wild-type dimeric Cks1 (yellow and blue). In dimeric Cks1, Arg33, Arg42, Ser82, and Trp85 are contributed by one molecule of Cks1 (yellow) while Arg102 is provided by the swapped ß-strand (blue).


a.
C
N
b.
C
N
c.
H91
A92
C90
124°
P93
C90
93°
A93
H91
A92
d.
S82
R102
R33
W85
R42

hinge loop HAP/AEPH (residues 91-96) (Figure 3.3c). The overall angle of the hinge loop is reduced by ~30 degrees, as calculated by measuring the angle defined by the Cα of Cys90, His91, and Ala92.

Cks proteins have been implicated in targeting cyclin-Cdk complexes to phosphoproteins via interaction with a conserved cationic pocket on Cks1. In the wild-type dimer fold, the anion-binding pocket of Cks1 is comprised of Arg33, Arg43, Ser82, and Trp85 from one molecule and Arg102 from the adjacent molecule. The structure of Cks1 P93A shows that in the monomer fold, as in CksHs1 and suc1, the anion-binding pocket is completely assembled from a single Cks1 molecule. Superposition of the anion binding pockets of dimeric Cks1 and Cks1 P93A shows that the positions of these residues are extremely similar (Figure 3.3d). Therefore, consistent with previous NMR data (Odaert et al. 2002), there is no structural evidence to conclude that changes in the hinge region are transduced to the phosphate binding pocket.

3.3.3. Comparison of Cks1 P93A with Other Monomeric Cks Proteins Reveals a Role for Pro93 in Cdk Binding

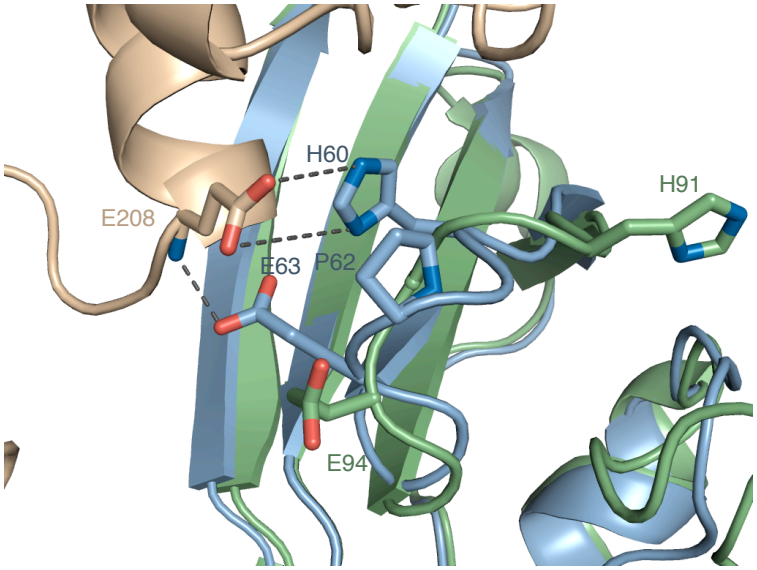
We found that unlike wild-type Cks1, Cks1 P93A does not form a stable complex with Cdk2 despite existing in the preferred conformation for Cdk binding. The structure of Cdk2 bound to CksHs1 shows that Pro62, the residue analogous to Pro93 in Cks1, does not form any direct contacts with Cdk in the binding interface (Bourne et al. 1996). We therefore questioned whether Cdk binding depends on Pro62/93 to position a key residue in the hinge region of

Cks1, and we searched for structural differences between the hinge regions of Cks1 P93A, monomeric wild-type suc1, and Cdk-bound CksHs1. Pro62 lies between two residues that make critical polar interactions with Cdk2. CksHs1 His60 forms a salt bridge with the Glu208 side chain of Cdk2, and CksHs1 Glu63 makes a hydrogen bond to the backbone amide proton of Glu208 (Figure 3.4a). In our structure of Cks1 P93A, His91 is oriented nearly 180 degrees rotated outward relative to Cdk-bound His60 of CksHs1 (Figure 3.4a, Figure 3.5), a position incompatible with forming contacts with Cdk2. We also observed a slight difference in the orientation of Glu94, another crucial Cdk binding residue (Figure 3.4a). However, unlike His91, Glu94 can be reoriented toward the Cdk interface by adopting a different rotamer conformation. While the loss of Pro93 predictably results in changes to the backbone dihedral angles of Glu94, there is only minimal displacement (0.89 Å) of the C of Cks1 Glu94 compared to the C of Cdk-bound CksHs1 Glu63. In addition, examination of the crystal lattice suggests that the position of Glu94 is influenced by crystal packing. Based on these observations, we predict that differences at Glu94 observed in the structures likely do not affect Cdk binding.

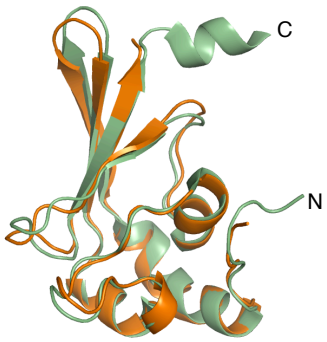
One possible alternative explanation for the differences in histidine positions in the unbound Cks1 P93A structure and the bound wild-type CksHs1-Cdk complex is that Cdk binding induces movement of the histidine to form the salt bridge with the Cdk glutamate. We therefore also compared the structure of Cks1 P93A with the crystal structure of the wild-type *S. pombe* homolog suc1,

Figure 3.4. Cks Hinge Residue Interactions with Cdk2 and Structural Comparison of the suc1 and Cks1 P93A Monomers. (a) Comparison of Cks1 P93A (green) and Cdk2-bound CksHs1 (blue) (PDB ID: **1BUH**) shows a significant displacement of His91 due to the P93A substitution. Glu208 of Cdk2 (light brown, sticks) forms a salt bridge with CksHs1 His60 but could not contact His91 in Cks1 P93A. The difference in rotamer conformations of CksHs1 Glu63, which also makes an important contact with the backbone amide of Glu208, and Cks1 P93A Glu94 is likely due to crystal packing. (b) Alignment of the suc1 (orange, coordinates communicated by J. Endicott) and Cks1 P93A monomer structures. (c) Close-up of the hinge region reveals that His88 of the free suc1 monomer (orange) and His60 of CksHs1 monomer bound to Cdk2 (blue) have a similar position. In contrast, His91 of the free Cks1 P93A monomer (green) is displaced. This comparison demonstrates that the histidine conformation is not induced to interact with Cdk2 upon Cks-Cdk2 association.

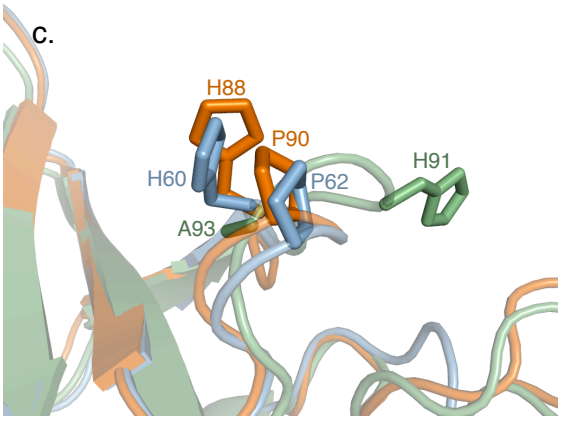
a.



b.



c.



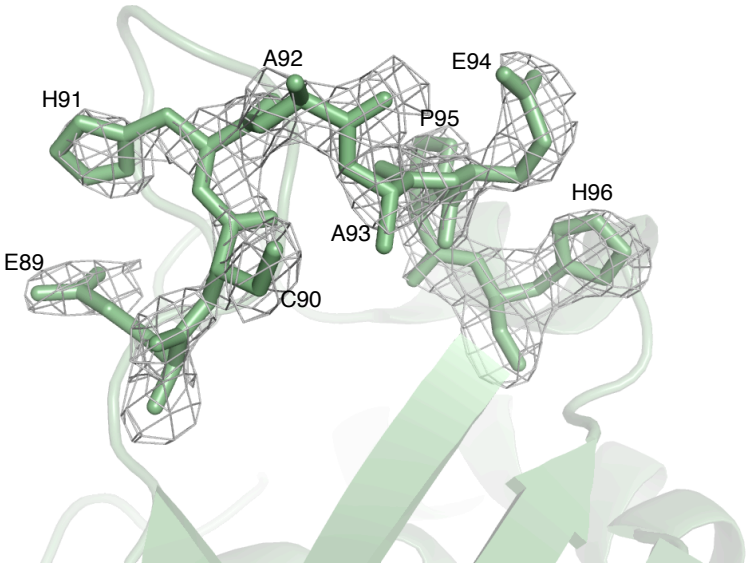


Figure 3.5. Electron Density Map of the Cks1 P93A Hinge Region. A simulated annealing omit (F_o-F_c) electron density map was generated using Phenix (gray mesh, contoured at 2.5σ (McCoy et al. 2007). The hinge region (residues 89-97) of Cks1 P93A was deleted from the input model.

which was also solved as a monomer and unbound to Cdk (Endicott et al. 1995). Superposition of Cks1 P93A with suc1 reveals that the two Cks proteins are almost structurally identical (rmsd = 0.766 Å over 83 C; Figure 3.4b). However, as in the structural comparison with CksHs1-Cdk2, His88 in suc1 is positioned differently from His91 in Cks1 P93A and is pointing properly towards the Cdk interface. That His88 of unbound suc1 is oriented similarly to His60 of Cdk-bound CksHs1 suggests that Cdk binding *per se* is not responsible for the arrangement of this residue (Figure 3.4c). In sum, comparison of the Cks1 P93A structure with wild-type structures of Cdk-bound and unbound Cks monomers

reveals that Cks1 Pro93 is essential for positioning His91 properly for Cdk binding.

To confirm the importance of His60/His91 in Cdk binding directly, we expressed and purified CksHs1 H60A and Cks1 H91E and performed ITC experiments with Cdk2 as described earlier (Figure 3.1). Both CksHs1 H60A and Cks1 H91E failed to bind Cdk2, suggesting that as previously observed for Glu63/Glu94 (Bourne et al. 1996), this residue is essential for Cdk binding. CD results verify that this failure to bind is not due to unfolding due to the introduced mutation (Figure 3.6). This result is consistent with the role for His60 at the Cdk-Cks interface revealed by the structure of Cdk2/CksHs1 and together with the P93A structure supports our hypothesis that Pro62/Pro93 is critical for Cdk binding because it properly positions His60/91.

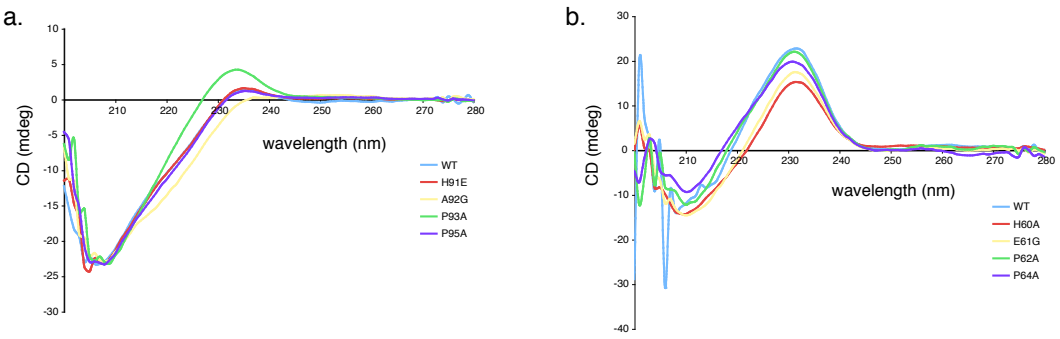


Figure 3.6. CD Results. (a) CD spectra of S. cerevisiae Cks proteins under conditions used in binding experiments. (b) CD spectra of human Cks proteins. Spectra were baseline corrected using buffer measurements and normalized to demonstrate similar spectral shapes. Although relatively weak, signals for CksHs1 and mutants are observed with a minimum ~210-230 nm. The maximum at 235 nm has been observed for CksHs1 elsewhere (Seeliger et al. 2002).

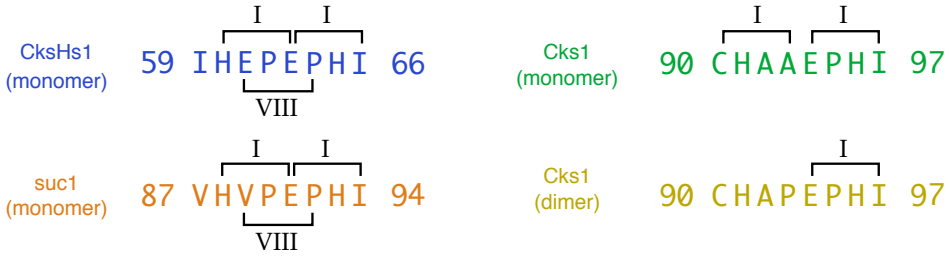
3.3.4. Cks1 Pro93 Stabilizes a β-turn Network in the Monomer That Is Critical for Cdk Binding

Prolines are commonly found in hinge regions of proteins that undergo domain swapping, and the constraints they impose on backbone conformation and dynamics are thought to be critical for tuning monomer-dimer equilibria (Bergdoll et al. 1997). In Cks proteins particularly, it has been suggested that backbone strain induced by the two hinge prolines dictates the hinge conformation in the monomer and dimer structures (Rousseau et al. 2001; Schymkowitz, Rousseau, and Itzhaki 2000). We questioned whether backbone rigidity also explains the requirement of Pro62/Pro93 for Cdk binding by influencing the orientation of His60/His91. Ramachandran analysis indicates that proline especially restricts the conformational space of its preceding residue (Schimmel and Flory 1968). We therefore introduced a glycine substitution to the residue preceding Pro62/Pro93 and performed our ITC assay with Cdk2. Both CksHs1 E61G and Cks1 A92G were able to bind Cdk2 essentially identical to wild-type CksHs1 and Cks1, respectively (Table 3.1). Therefore, the function of Pro62/Pro93 in positioning His60/His91 does not depend on influencing the backbone rigidity of the intervening residue.

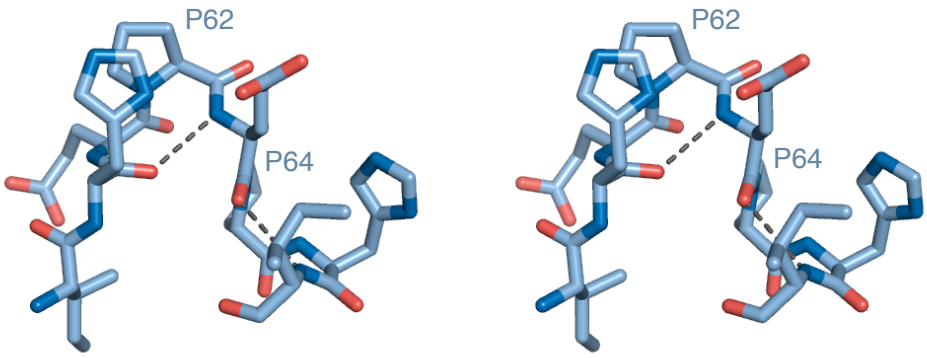
We found an alternate explanation for the role of Pro93 in His91 positioning and Cdk binding through comparison of the Cks1 P93A and wild-type Cks protein hinge structures (Figure 3.7). In the monomeric CksHs1 and suc1 structures, the hinge residues form a compact structure consisting of three

Figure 3.7. **Comparison of Cks Hinge Region β-turn Structures.** (a) Hinge region sequences and schematic representation of corresponding turn structures. (b) The hinge region of CksHs1 (stereo view, blue) consists of an (*I*, *I* +1, *I*+3) triple turn, in which the first and third turns are type I and the second turn is type VIII. The structure of the suc1 monomer is similar but not shown here. (c) The hinge region structure of Cks1 P93A monomer (stereo view, green) consists of two separate type *I* turns. (d) The hinge region structure of the wild-type Cks1 dimer (stereo view, yellow) is relatively extended and contains a single type I turn.

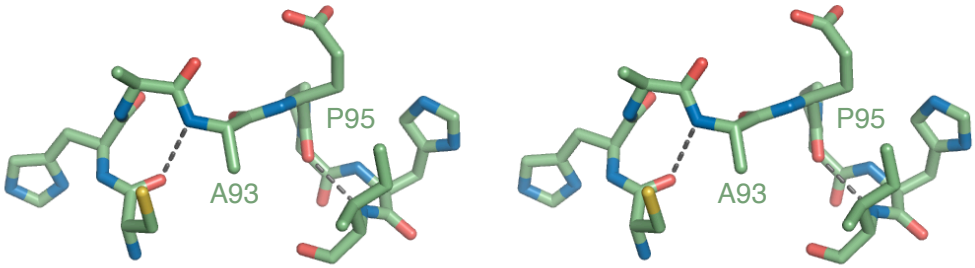
a.


CksHs1
(monomer)
59 IHEPEPHI 66
I I
VIII
Cks1
(monomer)
90 CHAAEPHI 97
I I
suc1
(monomer)
87 VHVPEPHI 94
I I
VIII
Cks1
(dimer)
90 CHAPEPHI 97
I

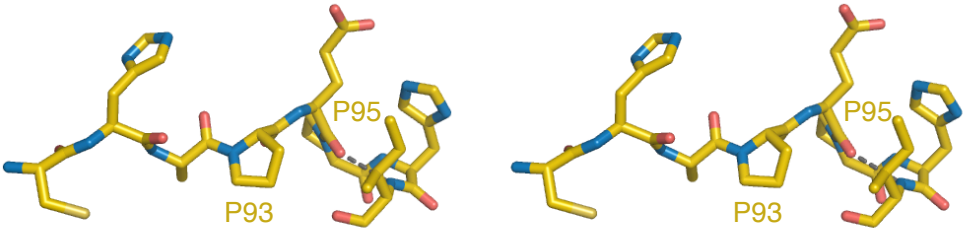
b.


P62
P64
P62
P64

c.


P95
A93
P95
A93

d.


P95
P93
P95
P93

β-turns (Figure 3.7a and 3.7b). Analysis of the backbone dihedral angles indicates that the structure is formally an (*I*, *I*+1, *I*+3) triple turn, in which the *I* and *I*+3 turns are type I and the *I*+1 turn is type VIII (Hutchinson and Thornton 1994). The critical His60 (CksHs1 numbering) is in the *i* position of the first turn, and its carbonyl oxygen forms a hydrogen bond with the amide hydrogen of Glu63 in the *i*+3 position. This conformation orients both the His60 and Glu63 side chains to form a common interface for Cdk binding. In the Cks1 P93A structure (Figure 3.7c), the hinge conformation is altered such that the *I* and *I*+1 turns of the triple turn are lost and a new type I turn is formed with Cys90 in position *i* and the mutated residue Ala93 in position *i*+3. His91 is in the *i*+1 position of the new turn, and, accordingly, the side chain is oriented in a direction away from the Cdk binding interface.

Structural analysis suggests several reasons for the reorganization of the hinge region turn composition and repositioning of His91. First, the P93A mutation disfavors formation of the (*I*, *I*+1) double turn because proline is highly preferred in the *i+1* position of type VIII β-turns (Hutchinson and Thornton 1994). Second, van der Waals contacts between the Pro and the His side chains are lost upon mutation of the proline to alanine (Figure 3.7b). Third, the P93A mutation creates an amide hydrogen that stabilizes the Cys90-Ala93 type I turn through formation of a hydrogen bond with the Cys90 carbonyl (Figure 3.7c). Finally, in the P93A mutant, His91 is stabilized in the *i*+1 position of the new turn by formation of a salt bridge between its side chain and the side chain of Glu89.

This interaction further orients the His91 side chain away from the Cdk binding interface. The Cys90-Ala93 type I turn also offers an alternative mechanism to backbone strain for how the P93A mutation stabilizes the monomer conformation. In the wild-type dimer conformation (PDB ID: **1QB3**), Cys90-Pro93 are in an extended conformation that stretches towards the dimerization partner to swap the following β-strand (Figure 3.7d). Therefore, dimerization of Cks1 P93A is likely disfavored, because forming the required extended structure requires breaking the stable Cys90-Ala93 turn.

3.4. Discussion

Cks proteins have served as an informative system for understanding domain swapping *in vitro*, and there is a rich literature characterizing the structural role of conformational strain within the hinge region as influencing the mechanism and thermodynamics of dimerization (Bader et al. 2006; Rousseau et al. 2001; Schymkowitz, Rousseau, and Itzhaki 2000; Seeliger, Breward, and Itzhaki 2003; Seeliger et al. 2002; Seeliger et al. 2005). However, the biological relevance, if any, of Cks domain swapping remains unknown. Considering the structural evidence that only the monomer conformation of Cks is compatible with Cdk binding, the most often proposed model has been that the monomer-dimer equilibrium regulates Cks-Cdk association and thereby regulates Cdk function. We aimed to study the Cks1 dimer in budding yeast, but could not detect it by coimmunoprecipitation from asynchronously growing cells (Figure 3.8). While our result is negative, it at least suggests that the existence of

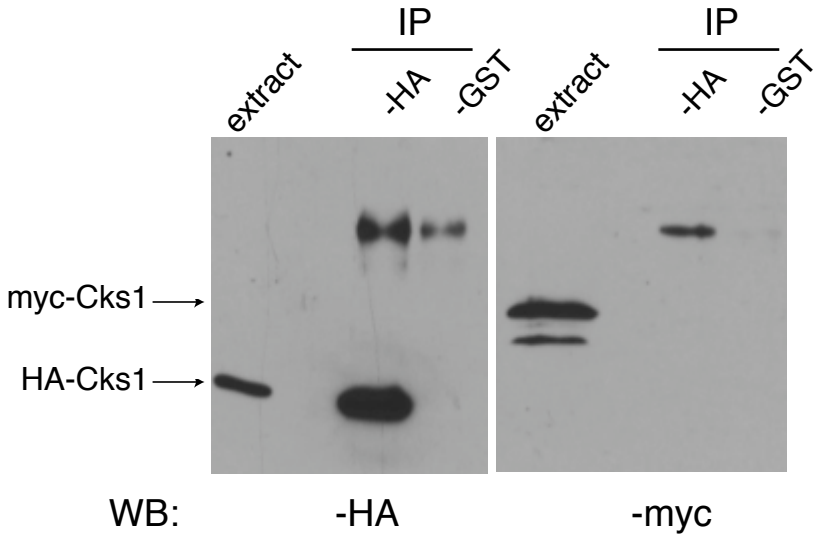


Figure 3.8. Cks1 Dimers are Undetectable in Asynchronous Budding Yeast Extracts. Cks1-3xHA fails to immunoprecipitate Cks1-13xMyc. Following 3 hour galactose induction, supernatants from yeast expressing both Cks1-3xHA from the *GAL1* promoter and Cks1-13xMyc from the endogenous *CKS1* promoter were analyzed directly (extract) or following either anti-HA or anti-GST precipitation by Western blotting with the indicated antibody.

dimeric Cks in budding yeast may be limited to a specific functional context such as a precise cell cycle regulatory step.

A strong motivation for postulating the functional relevance of domain swapping *in vivo* is the invariant conservation of hinge residues Pro62/93 and Pro64/95 (Schymkowitz, Rousseau, and Itzhaki 2000). These prolines tune the dimerization equilibrium *in vitro*, but unlike other hinge residues, they do not interact directly with Cdk (Bourne et al. 1996). Here we tested whether proline mutations modulate Cdk binding in the expected manner and instead found that a P62/93A mutation, which stabilizes the monomer, results in a loss of Cdk binding. The crystal structure of Cks1 P93A shows that Pro93 is required for

positioning His91 properly for Cdk binding. We propose that Pro93 is the lynchpin in the formation of a (*I*, *I*+1) double β-turn structure in the wild-type Cks1 monomer fold, and that the alanine substitution promotes a different, thermodynamically favored β-turn that precludes Cdk binding. Furthermore, we suggest that in light of the formation of a new β-turn that should inhibit strand exchange, there is no need to invoke arguments involving loop strain generated in the peptide bonds preceding hinge prolines to explain Cks domain swapping. Our alternate explanation for the stability of the Cks1 P93A monomer fold is corroborated by the fact that glycine substitution at the residue preceding Pro62/Pro93 has no effect on Cdk binding and hence must not cause significant rearrangement of the hinge region, despite relieving positional backbone rigidity. Therefore, our results indicate that the conservation of hinge prolines and their structural properties in Cks oligomerization may instead reflect their critical roles in the formation of the Cks-Cdk interface.

3.5. Materials and Methods

3.5.1. Protein Expression and Purification

S. cerevisiae Cks1 and Cks1 P93A were expressed from a pET vector in *E. coli*. Expression was induced with 1 mM IPTG overnight at room temperature. Cells were harvested and lysed in 25 mM Tris pH 8.0, 1 mM DTT, and 1 mM PMSF. Clarified lysates were flowed over DEAE sepharose resin, washed with lysis buffer, and eluted with lysis buffer + 100 mM NaCl. Cks1 was then purified by Source 15Q (GE Healthcare) cation exchange chromatography at pH 8.0 followed

by size-exclusion chromatography. All Cks1 proteins were full length with the exception of Cks1 P93A, which was truncated at residue 117. Experiments show that the poorly conserved C-terminal polyglutamine tail (residues 118-133) of Cks1 is not required for its biological function, including Cdk binding (Bourne et al. 2000). *S. cerevisiae* Cks1 H91E, A92G, and P95A and all human CksHs1 proteins were expressed as GST-fusion proteins in *E. coli* and purified with glutathione sepharose chromatography. Following elution from glutathione sepharose, the GST tag was cleaved using GST-TEV protease (1-2% by weight). The free GST tag and GST-TEV were removed during subsequent purification using cation exchange chromatography at pH 6.0 (CksHs1) or Source 15Q anion exchange chromatography at pH 8.0 (Cks1 mutants) followed by a second glutathione sepharose column. All mutant proteins were confirmed to be folded using CD (Figure 3.6). Oligomerization states of Cks proteins were analyzed using size-exclusion chromatography performed at concentrations comparable to binding experiment conditions (Figure 3.2). Human Cdk2 was expressed with an N-terminal hexahistidine tag for 2-3 days using a baculovirus vector in Sf9 cells (Invitrogen) in suspension in Sf900 II media (GIBCO). 6xHis-Cdk2 was purified by Ni^{2+}-NTA affinity chromatography. Initial ITC experiments were performed with Cdk2 from which the 6xHis tag was cleaved overnight at 4°C using TEV protease (1-2% by weight) following elution from the nickel column. However, it was determined that the presence of the 6xHis tag had no effect on Cks binding, and subsequently TEV cleavage was not performed.

3.5.2. Isothermal Titration Calorimetry

Experiments were performed using a VP-ITC instrument (Microcal). Purified proteins were dialyzed overnight at 4ºC in 25 mM Tris pH 8.0, 150 mM NaCl. In a typical experiment, Cks proteins at a concentration of ~0.2 -1 mM were titrated into a 20-90 μM solution of Cdk2. When binding was detected, experiments were performed in duplicate. Binding constants were calculated by averaging the K_ds and the error is the standard deviation of the K_ds.

3.5.3. Analytical Size Exclusion Chromatography

Cks proteins were dialyzed overnight in 50 mM sodium phosphate, 300 mM NaCl (pH=6.8). Proteins were then concentrated as necessary and loaded at a volume of 0.5 ml onto a Superdex 75 HR10/30 analytical gel filtration equilibrated in dialysis buffer.

3.5.4. Crystallization and Structure Determination

Prior to crystallization, Cks1 P93A was purified using a Superdex75 column (GE Healthcare) equilibrated in 25 mM Tris pH 8.0, 200 mM NaCl, and 1 mM DTT. 25 mg/ml Cks1 P93A was mixed at a 1:1 ratio with a solution consisting of 0.1 M sodium cacodylate pH 6.5, 0.2 M sodium citrate, and 5% isopropanol. Crystals were grown by the hanging-drop vapor diffusion method. Data collection was performed at beamline 5.0.1 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory. Data were processed using Mosflm and SCALA (CCP4 1994; Leslie 2006). Crystals contained two monomer Cks1 molecules in the asymmetric unit. The structure was determined by

molecular replacement with PHASER (McCoy et al. 2007) using two molecules of dimeric Cks1 lacking the hinge loop (residues 6-88, 94-105) as a search model (PDB ID: **1QB3**). A simulated-annealing F_o-F_c map was generated to verify the positions of the hinge region residues (Figure 3.5). Residues 106-117 of molecule A and 1-6 and 112-117 of molecule B are not visible in the electron density map and presumed to be disordered in the crystals. The model was refined using iterative cycles of PHENIX and COOT (Adams et al. 2010; Emsley and Cowtan 2004). A citrate molecule was built in at occupancy = 0.5 using COOT and refinement was continued using refmac (CCP4 1994).In our model, 97.09% of the residues are in the preferred region of the Ramachandran plot, 1.94% in the additional allowed region, and 0.97% are outliers. Ramachandran outliers were identified as Ser82 of molecule A and Asp36 of molecule B.

3.5.5. Circular Dichroism

CD spectra were recorded on an Aviv 62DS CD spectrophotometer using a 1 mm pathlength quartz cuvette. The data were recorded every 1 nm using an integration time of 8s per step. Protein concentrations were 2.5-25 μM in 25 mM Tris pH 8.0, 150 mM NaCl. The results shown here are the average of three experiments for each sample.

3.5.6. Yeast Strains, Plasmids, and Culture Conditions

Yeast strains are derivatives of the W303-1A strain background (*ade2-1 can1-100 ura3-1 leu2-3,112 his3-11,15 trp1-1*) and are listed in Table 3.3.

Name	MAT	Relevant Genotype	Source
DK186	a	bar1	Doug Kellogg
SH1012	a	bar1 CKS1-13xMyc::His3MX6	This study
EM8	a	bar1 CKS1-13xMyc::His3MX6 URA3:GAL1-CKS1-3xHA	This study

Table 3.3. Strains Used in This Study

Standard genetic and molecular biology methods were used for strain construction. To create a strain expressing Cks1-13xMyc under the endogenous Cks1 promoter (SH1012), PCR was used to amplify pFA6a-13Myc-His3Mx6,[6] and the product was transformed into DK186. To construct an integrating plasmid that expresses Cks1-3xHA from the *GAL1* promoter, the *CKS1* open reading frame without the stop codon was amplified and cloned into the *BamH*I and *Eag*I sites of the *GAL1-3xHA*-C-terminal tagging vector pSH32A to create pSH108A. EM8 was made by digesting pSH108A with *Stu*I to target integration at the *URA3* locus in SH1012. Oligonucleotide sequences are available upon request.

3.5.7. Coimmunoprecipitation of Cks1-3xHA and Cks1-13xMyc

SH1012 cells expressing Cks1-3xHA from the *GAL1* promoter and Cks1-13xMyc from the endogenous Cks1 promoter were lysed by bead beating in lysis buffer (50 mM Hepes-KOH pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 5% glycerol, 0.15% Tween-20, 2.0 mM PMSF). Cell extract was centrifuged for 5 minutes at 13,800 x g. 10 µl of extract was removed for "extract" samples. Extracts were then incubated with 15 µl of protein A beads loaded with 10 µg of either rabbit anti-HA or rabbit anti-GST (negative control) antibodies. Extracts were incubated

with beads for 2 hours at 4°C. Following binding, beads were washed three times with 500 µl cold lysis buffer without PMSF or Tween-20. Washed beads were pelleted by brief centrifugation and resuspended in 50 µl of protein loading buffer (65 mM Tris HCl pH 6.8, 3% SDS, 10% glycerol, 5% ß-mercaptoethanol, 50 mM NaF, 100 mM ß-glycerophosphate). 40 µl of loading buffer was added to the extract samples. Samples were boiled for five minutes, analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with either mouse anti-HA or mouse anti-Myc antibodies. Bands were visualized by enhanced chemiluminescence.

3.6. Accession Numbers

Coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID: **3QY2**).

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Chapter 4: Determining Optimal Phosphopeptide Determinants of Cks1 Binding

4.1. Introduction

Progress through the cell cycle is mediated by cyclin-dependent kinases (Cdks). Cdks are responsible for the phosphorylation of hundreds of cell-cycle stage specific targets (Ubersax et al. 2003; Holt et al. 2009). The phosphorylation of these substrates at specific sites and times underlies the ability of the cell to execute a multitude of discrete yet coordinated events following the activation of a single enzyme.

Cdk activity is regulated by a variety of mechanisms: (1) Cyclin binding both activates the Cdk and affects its substrate choice (Murray 2004); (2) Inhibitors inactivate Cdks at key points, such as mitotic exit or prior to S-phase entry (Sherr and Roberts 1999); (3) Cdks are themselves phosphorylated by both activating (by CAK) and inactivating (by Wee1, for example) enzymes (Kaldis 1999; Gould and Nurse 1989) and (4) Cdks are directly associated with a small protein called Cks1, whose functions in Cdk regulation are still largely unknown (Hadwiger et al. 1989; Pines 1996).

Cks1 has a conserved positively charged pocket that is capable of binding phosphates and phosphoproteins (Arvai et al. 1995; Kõivomägi, Valk, Venta, Iofik, Lepiku, Balog, et al. 2011). Recent work supports a long-standing yet unverified hypothesis that one function of Cks1 is targeting Cdks to phosphoprotein substrates (Bourne et al. 1996; Kõivomägi, Valk, Venta, Iofik, Lepiku, Balog, et al.

2011). This hypothesis is attractive in light of older research showing that Cks1 promotes hyperphosphorylation of a number of multiply phosphorylated Cdk substrates (Patra et al. 1999). We propose a model in which initial or "priming" phosphorylation, catalyzed by Cdks or other kinases, creates Cks1 docking sites on multiphosphorylated substrates, tethering Cdks to their substrates and accelerating further phosphorylation (Figure 4.1).

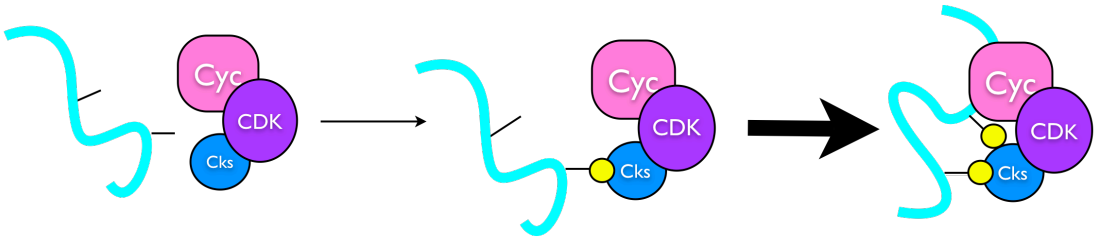


Figure 4.1. The Tethering Model of Cks1-Mediated Multisite Phosphorylation. Binding between substrate and Cdk at a distal docking site induces proximity of additional phosphoacceptor sites, decreasing their effective K_m and perhaps influencing site choice and enzyme processivity.

To investigate this model and its predictions, we needed to choose representative Cdk substrates. We used the following criteria in our selection: (1) the substrate must be multiply phosphorylated; (2) the substrate should already be known to form a stable, preferably phosphorylation-dependent complex with Cdk; and (3) for ease of future genetic experiments, the above criteria must be satisfied in budding yeast. Three *S. cerevisiae* Cdk1 substrates that met these criteria were Cdc6, Swe1, and Sic1 (Mimura et al. 2004; Harvey et al. 2005; Schneider, Yang, and Futcher 1996).

Here we define for the first time the molecular correlates of Cks1-phosphoprotein binding. Using the Cks1 phosphoprotein consensus motif, we introduce a subset of Cdk substrates whose phosphorylation is may be Cks1-directed. Finally, we elucidate important details of functional interactions between Cdks and their inhibitors that are mediated by Cks1, assigning Cks1 a molecular function in Cdk regulation that validates its conservation and its requirement throughout the cell cycle.

4.2. Results

4.2.1. Qualitative Assays for Cks1 Binding

We asked whether Cks1 is generally involved in the formation of phosphorylation-dependent stable complexes between Cdk and its substrates. First we performed a qualitative bead-based pulldown assay for Cks1 binding. Purified His_6-Cdc6 NTD or His_6-Swe1 NTD was phosphorylated by recombinant CycA/Cdk2. The extent of phosphorylation was measured by mass spectrometry and was also observable as an electrophoretic mobility shift. His_6-Cdc6 NTD phosphorylation was complete, phosphorylating all four Ser/Thr-Pro sites within the construct. Only phosphorylated His_6-Cdc6 NTD is able to pull down GST-Cks1 (Figure 4.2). His_6-Swe1 NTD phosphorylation was incomplete (up to three phosphates added although eight Ser/Thr-Pro motifs are available). Incomplete phosphorylation of Swe1 NTD may indicate that despite the usual promiscuity of CycA/Cdk2, Swe1 is a poor CycA/Cdk2 substrate. However, because quantities of Clb2/Cdk1 were limiting, CycA/Cdk2-phosphorylated

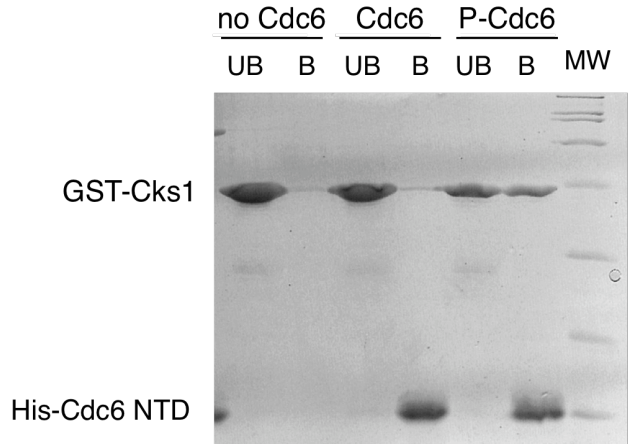


Figure 4.2. Cks1 Binds Phosphorylated Cdc6 NTD. GST-Cks1 and either no (lanes 1-2), unphosphorylated (lanes 3-4) or phosphorylated (lanes 5-6) His_6-Cdc6 NTD were incubated with Ni-NTA beads. Beads were then washed and eluted and proteins from unbound and bound fractions were analyzed by SDS-PAGE and visualized with Coomassie stain. *Credit: Seth Rubin*

Swe1 was used in pulldown assays. Despite incomplete phosphorylation, phosphorylation-dependent Cks1 binding was also observed for His_6-Swe1 NTD (Figure 4.3).

Having identified multiply phosphorylated Cdk substrates with affinity for Cks1, we next attempted to identify what, if any, sequence context contributes to the specificity of Cks1-phosphosubstrate binding. A series of His_6-Cdc6 constructs were cloned and expressed to examine the effects of removing one or more phosphorylation sites on Cks1 binding (Figure 4.4). Cdc6 NTD phosphorylated only at Thr7 is sufficient to pull down Cks1, while Cdc6 NTD phosphorylated at the three remaining sites fails to pull down Cks1 (Figure 4.5). It is important to note that the construct labeled “Triple Mut” in Figure 4.5 is actually only a double mutant, as S43 was reverted back to “S” in an unfortunate

cloning mishap. True Cdc6 Triple Mut was cloned and sequenced by Will Finch in May 2009 and should exist somewhere labeled as "Tmut3 mini A."

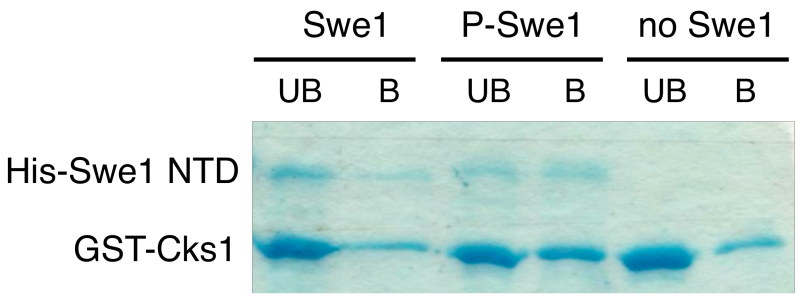


Figure 4.3. Cks1 Binds Phosphorylated Swe1 NTD. GST-Cks1 and either unphosphorylated (lanes 1-2), phosphorylated (lanes 3-4) or no (lanes 5-6) His_6-Swe1 NTD were incubated with Ni^{2+}-NTA beads. Beads were then washed and eluted and proteins from unbound and bound fractions were analyzed by SDS-PAGE and visualized with Coomassie stain.

4.2.2. Quantitative Assays for Cks1 Binding

We used isothermal titration calorimetry (ITC) to measure the binding constants for Cks1 and phosphorylated NTD constructs. ITC experiments

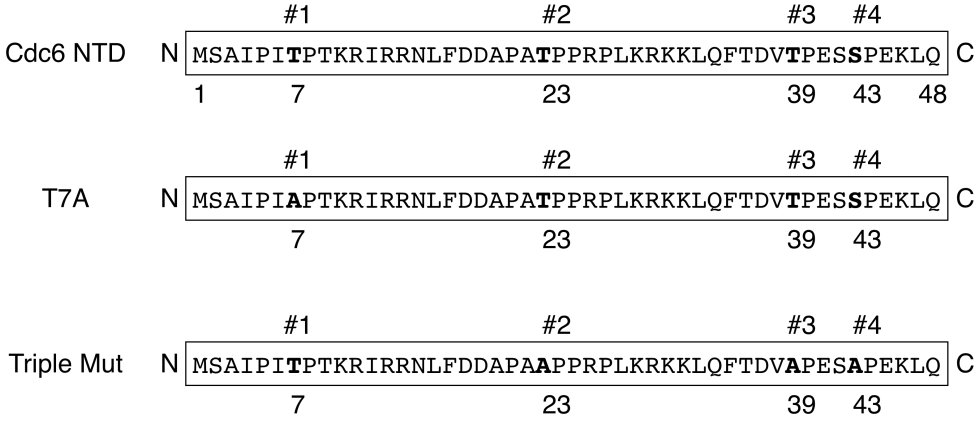


Figure 4.4. Cdc6 Constructs Used in This Study. Note that "Triple Mut" is correctly presented here, but S43 remains a serine in our experiments.

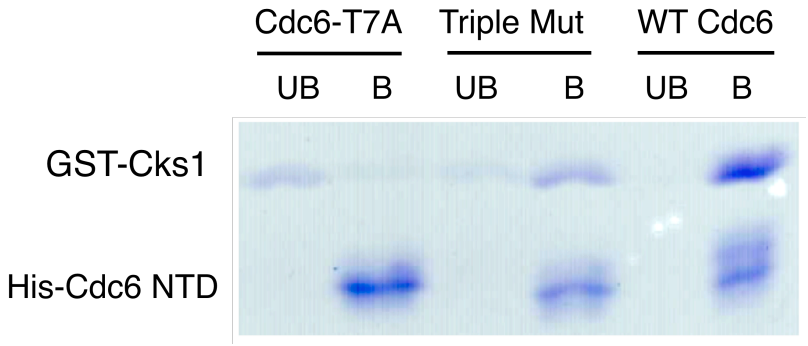
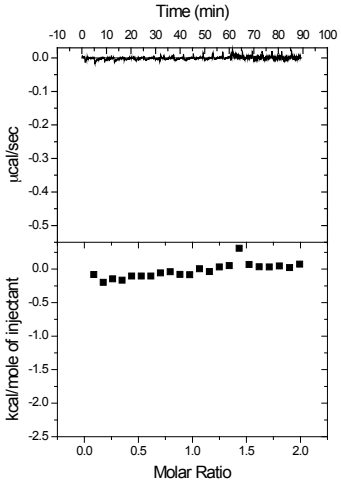


Figure 4.5. Phosphorylated T7 is Necessary and Sufficient for Cks1 Pull-down with Cdc6 NTD. GST-Cks1 and either Cdc6 pT7A (lanes 1-2), Cdc6 Triple Mut (lanes 3-4) or wild-type Cdc6 NTD (lanes 5-6) were incubated with Ni^{2+}-beads. Beads were then washed and eluted and proteins from unbound and bound fractions were analyzed by SDS-PAGE and visualized with Coomassie stain. *Credit: Alexander Hirschi*

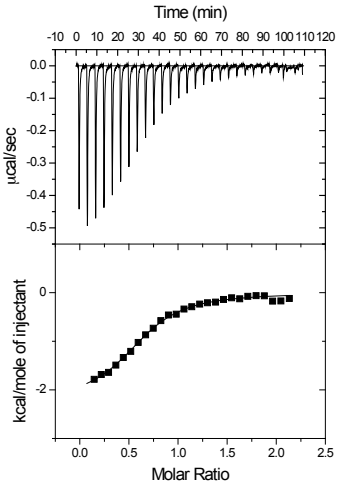
confirmed that Cdc6 NTD pT7 binds Cks1 with similar affinity as fully phosphorylated Cdc6 NTD (Figure 4.6). However, there is some ambiguity in the ITC results that further experiments may clarify. In one experiment, pCdc6 T7 fails to bind Cks1, while the same experiment repeated shows pCdc6 T7 binding Cks1 with $K_d \sim 40$ μM, which is reduced but not elimination of binding. It is important to note that the Swe1 NTD used in this experiment is probably not fully, or possibly even multiply, phosphorylated. Additionally, this experiment was only performed once, and the stoichiometry is troubling, suggesting protein concentration measurement problems. We narrowed the sequence sufficient for Cks1 binding to the nine residue peptide SAIPI{pT}PTK. Interestingly, the sequence context of the Swe1 NTD Cdk site T196 is very similar to that of Cdc6 T7 (RIPE{pT}PVK), but this phosphopeptide fails to bind Cks1 in an ITC experiment.

Figure 4.6. ITC Traces for Cdc6 and Swe1 Binding to Cks1. Unphosphorylated Cdc6 NTD fails to bind Cks1, while phosphorylated Cdc6 NTD binds with K_d = 10 μM. Phosphorylated Swe1 NTD bound with K_d = 26 μM; see caveats in text. AMH = Alexander M. Hirschi; SMR = Seth M. Rubin.

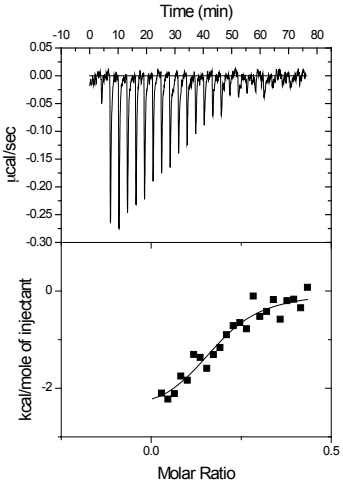
WT Cdc6 NTD



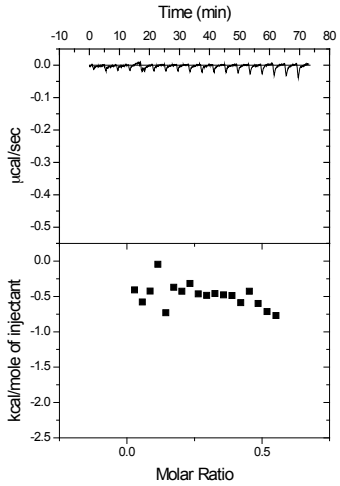
WT pCdc6 NTD



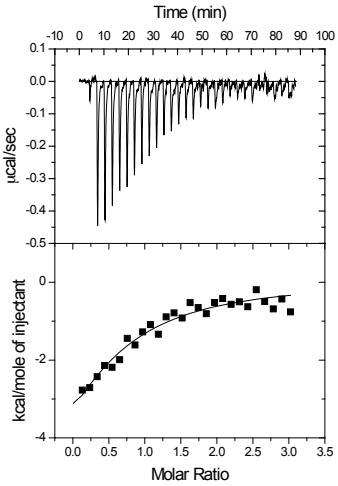
pCdc6 Triple Mut



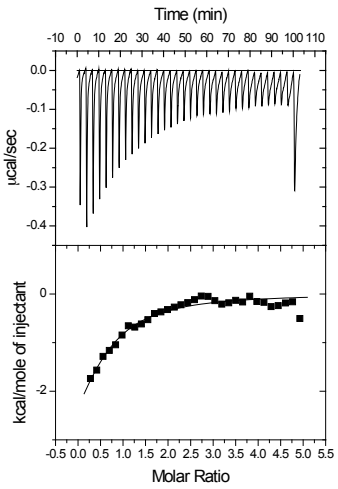
pCdc6 T7A (AMH)



pCdc6 T7A (SMR)



pSwe1 NTD



4.2.3. Identification of a Minimum Cks1-Binding PhosphoCdc6 Sequence

To ascertain the shortest sufficient phospho-T7 peptide for Cks1 binding we adopted a spot blot approach. Synthetic phosphopeptides (Genscript) were covalently cross-linked to BSA carrier protein and directly spotted onto PVDF membrane. Membranes were probed with His_6-Cks1 that was subsequently detected using anti-His-HRP (Santa Cruz Biotech). His_6-Cks1 was chosen to minimize the potential for nonspecific effects due to a GST tag; at the time of this experiment, we did not have a useful yeast anti-Cks1 antibody, as the Santa Cruz Biotechnology anti-human Cks1/2 antibodies failed to detect *S. cerevisiae* Cks1. We have since acquired anti-yeast Cks1 antibody from Mart Loog, although at the time of this writing it has not been tested in our hands. The SCBT anti-His-HRP antibody also generates vastly cleaner blots than the Kellogg lab's anti-GST

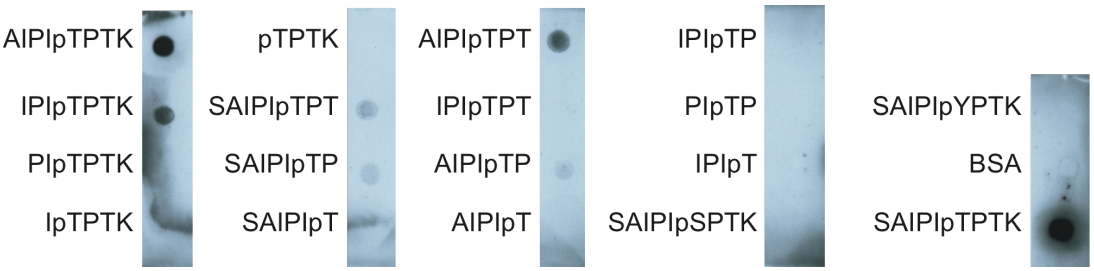


Figure 4.7. Cks1 Binds Truncated Phosphopeptides Derived from Cdc6 T7 Sequence. The shortest peptide capable of binding Cks1 was six residues; however; the 6-mer IPIpTPT failed to bind, suggesting specific sequence requirements may be more important than length.

primary. The resulting spot blot should be interpreted as qualitative as the peptide starting material was crude purity grade and there was no way to control for efficiency of BSA cross-linking. The shortest Cdc6 phospho-T7 peptide

sufficient for Cks1 binding was the 6-mer AIPI{pT}P (Figure 4.7). Within the same experiment we also demonstrated that substitution of phosphoserine and phosphotyrosine for phosphothreonine in the Cdc6 T7 sequence context eliminates Cks1 binding. An immediate priority for future work is to corroborate these results quantitatively using calorimetry.

4.2.4. Identification of a Cks1 Consensus

To identify the sequence determinants of Cks1 phosphopeptide binding, we used Spots peptide arrays (Richard Cook, MIT Biopolymers Laboratory). Each position of the 8mer Cdc6 T7 (SAIPI{pT}PTK) was altered to each of the twenty natural amino acids. Cks1 contains one cysteine residue, Cys90. We observed the need to prevent false positives due to disulfide linkages in the oxidizing conditions of the experiment. Conservative mutant Cks1 C90S was generated using site-directed mutagenesis but failed to bind positive control

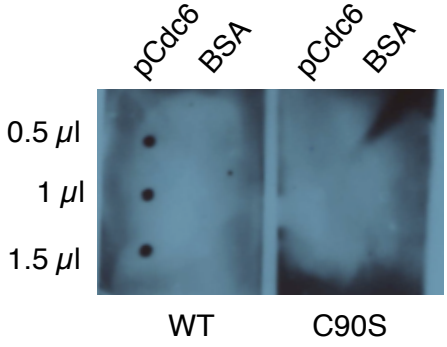


Figure 4.8. Cks1 C90S Fails to Bind Cdc6 T7 Phosphopeptide. PhosphoCdc6 T7 peptide conjugated to BSA or BSA alone was spotted in three different volumes onto each membrane. Membranes were blocked overnight and rocked for two hours at room temperature with 36 µg/ml wild-type His_6-Cks1 or His_6-Cks1 C90S in Blocking Buffer. Membranes were then washed and probed for one hour with 1:2000 anti-His_6-HRP antibody. Membranes were washed three times in TBS and developed with Supersignal chemiluminescence reagents.

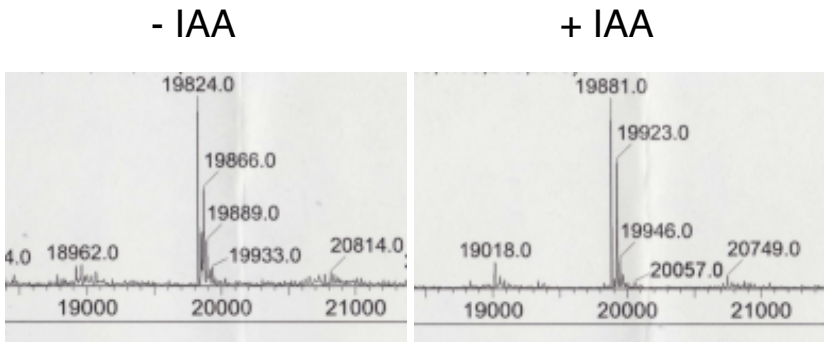


Figure 4.9. Mass Spectrometry of His_6-Cks1 and His_6-Cks1-al. His_6-Cks1 was treated with 10 mM DTT, followed by 25 mM IAA for 30 minutes in the dark, and then quenched with 20 mM DTT. Mass spectrometry shows a gain of 57 daltons, corresponding to a gain of $C_2H_3O_2$ and a loss of one proton.

pCdc6 T7 peptide conjugated to BSA (Figure 4.8). This is peculiar, given that Cys90 is in the hinge region between strands β3 and β4, on the opposite end of these strands from the anion-binding pocket. As an alternate approach, we chose to alkylate Cys90 using iodoacetic acid to generate His_6-Cks1-al. Alkylation was validated by mass spectrometry (Figure 4.9). Control experiments showed that, at least qualitatively, alkylation did not affect phosphopeptide binding. (Figure 4.10). Interaction of purified His_6-Cks1-al with peptide spots was detected using an anti-His_6 antibody conjugated to HRP.

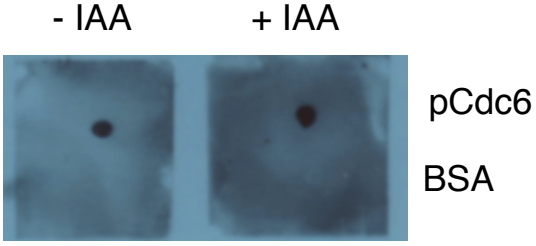
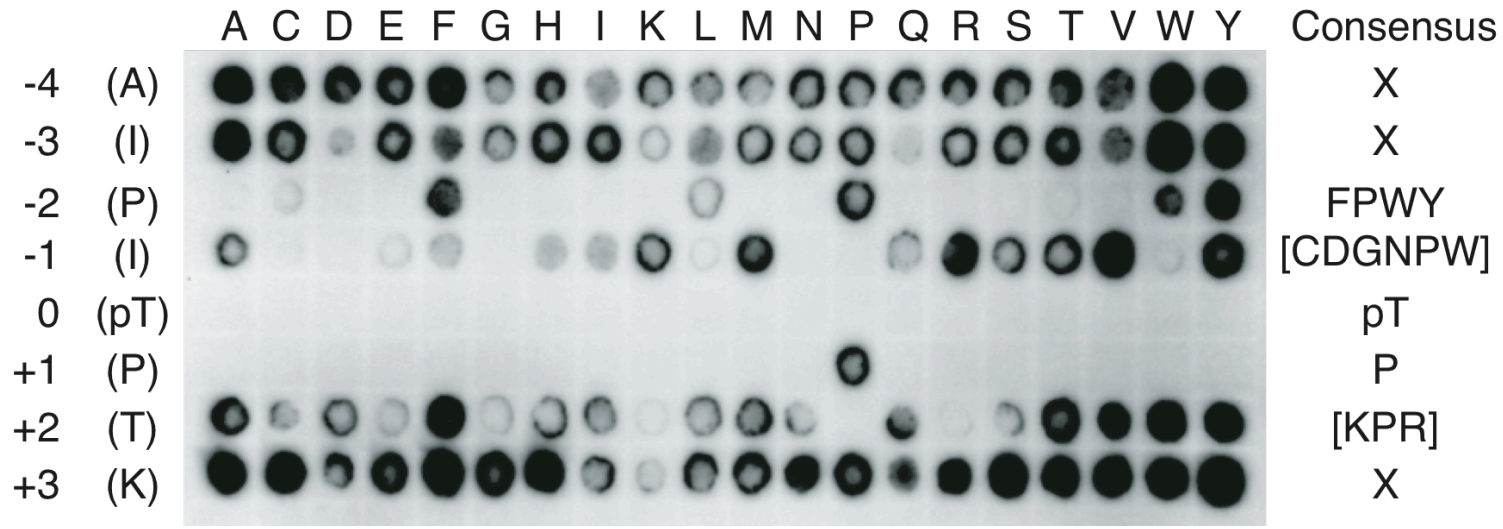


Figure 4.10. Alkylation Does Not Affect pCdc6 T7 Peptide Binding to His_6-Cks1. Membranes spotted with phosphoCdc6 T7 peptide conjugated to BSA and BSA were probed with His_6-Cks1 or His_6-Cks1-al. Binding was detected with anti-His_6-HRP antibody followed by chemiluminescence.

Analysis of duplicate array experiments revealed an absolute requirement for phosphorylated Thr and Pro at the +1 position (Figure 4.11). Bulky hydrophobic residues (F/P/W/Y) were strongly preferred in the -2 position. Pro was strongly disfavored in the +2 position, as were Arg and Lys to a lesser extent. Interestingly, basic residues were also disfavored at the +3 position, in contrast to their favorability in the Cdk consensus S/T-P-X-K/R. Several residues (C/D/G/N/P/W) were disfavored in the -1 position. The variant effects of substitution at the -1 position suggest interdependence or redundancy between the contributions of local sequence elements to Cks1 binding. Overall the substitution of bulky hydrophobics at the N- and C-termini of the peptide was preferred for binding. For search purposes we defined the Cks1 binding consensus as F/P/W/Y-[C/D/G/N/P/W]-pT-P-[P].

Figure 4.11. Characterization of the Cks1 Phosphopeptide Consensus Motif Using a Positional Scanning Array Based on Cdc6 T7. Each amino acid in the T7 sequence context (vertical axis) was systematically altered to each of the 20 natural amino acids (horizontal axis). The phosphorylated residue position is designated position 0 and the other positions are numbered -3 to +4 relative to position 0. The preferences exhibited by His-Cks1-al are listed on the right. Substitutions that are poorly tolerated are in square brackets and positions with no strong preferences are labeled with X.


A C D E F G H I K L M N P Q R S T V W Y
Consensus
-4 (A)
-3 (I)
-2 (P)
-1 (I)
0 (pT)
+1 (P)
+2 (T)
+3 (K)
X
X
FPWY
[CDGNPW]
pT
P
[KPR]
X

4.2.5. Searching Cdk Substrates for the Cks1 Consensus Sequence

The Cks1 binding consensus defined above was used to search the Yeast Genome Databank (www.yeastgenome.org) using the Pattern Matching tool (http://www.yeastgenome.org/cgi-bin/PATMATCH/nph-patmatch). The results of this search are summarized in Figure 4.12 and Appendix . A total of 896 sequences from 793 ORFs were identified. Of these, 128 matched Cdk substrates identified in one of two global Cdk1-substrate identification studies (Holt et al.

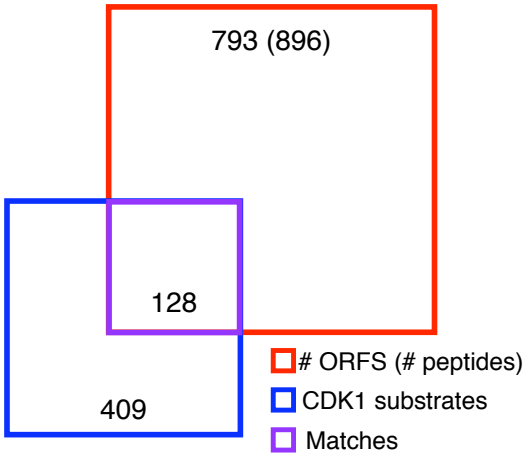


Figure 4.12. Schematic Representation of Pattern Match Results.

2009; Ubersax et al. 2003). This means that the Cks1 phosphobinding consensus was found in approximately 31% of putative Cdk1 substrates considered here (128/409). Twenty-nine proteins from the pattern search matched substrates identified in both studies, representing approximately 36% of the 80 shared substrates. Of 172 Cks1 physical and genetic interactors cataloged in BioGRID, 29 were also found in the pattern search results. A complete summary of the pattern matching results can be found in the Supplemental Excel File accompanying this

dissertation. Future experiments to define the Cks1 phosphopeptide binding consensus will start with the sequence context of the best Sic1 priming site, T5. It will be interesting to investigate the correlation between Cks1 binding affinity and functions such as phosphorylation site priming ability and Cks1-dependent Cdk inhibitory activity. Our collaboration with Mart Loog at the University of Tartu, Estonia provides a golden opportunity to explore these relationships kinetically.

4.2.6. Sic1

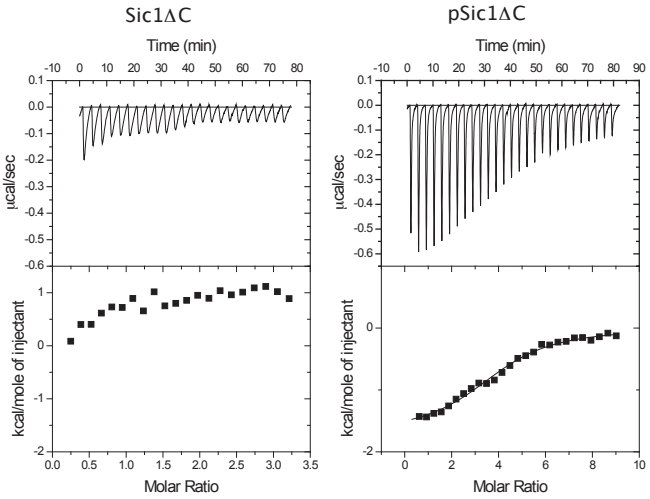
Concurrent with the above work, we collaborated with the Loog group to perform calorimetry experiments with Cks1 and phosphorylated Sic1 constructs. The Loog group had identified Cks1-dependent processive phosphorylation of Sic1 and pairs of primer/acceptor Cdk sites in the unstructured N-terminal domain of Sic1. They wanted to know which sites in Sic1 contributed to Cks1 binding, and how the sequence context of these sites affected affinity for Cks1. Sic1 constructs sent to us from the Loog group were amino acids 1-212 (Sic1ΔC). When phosphorylated constructs were sent, phosphorylation was assayed using PhosTag gels and samples were accompanied by emailed pictures of gels. As expected, unphosphorylated Sic1ΔC failed to bind Cks1 (Figure 4.13). Phosphorylated Sic1ΔC binds Cks1 with similar affinity to phosphorylated Cdc6 NTD. No single phosphorylation site was sufficient to bind with the same affinity as fully phosphorylated Sic1ΔC (Figure 4.14). The stoichiometry of binding for wild-type Sic1ΔC was between 3-4 Cks1 molecules per molecule of Sic1ΔC,

suggesting multiple weaker interactions are contributing to the appearance of higher affinity. Two series of experiments were performed. The first series focused on the sequence context of a single phosphorylation site, T33, because it was identified as a good priming site. The second looked at the ability of singly phosphorylated Sic1ΔC constructs to bind Cks1. Note that phosphoSic1ΔC T33 appears in both Figures 4.13 and 4.14; this is to illustrate that the original experimental conditions did not allow detection of weak binding because of erratic baseline behavior due to DTT. Table 4.1 summarizes the results of ITC experiments shown in Figures 4.13-14.

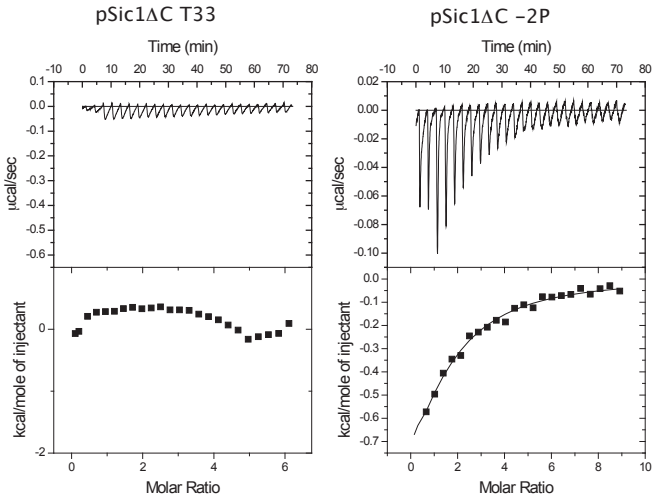
Sic1ΔC Construct	Cks1 K_d (μM)
pSic1ΔC	11 ± 2
pSic1ΔC T33	56 ± 12
pSic1ΔC -2P	23 ± 3
pSic1ΔC H1	20 ± 1
pSic1ΔC AB1	47 ± 9
pSic1ΔC T5	23 ± 13
pSic1ΔC T45	21 ± 4
pSic1ΔC S76	NB
pSic1ΔC T173	NB
pSic1ΔC T33S	NB
Sic1ΔC	NB
Sic1ΔC -2P	NB
Sic1ΔC T33S	NB

Table 4.1. Dissociation Constants for Cks1-Sic1ΔC Constructs. NB = no binding.

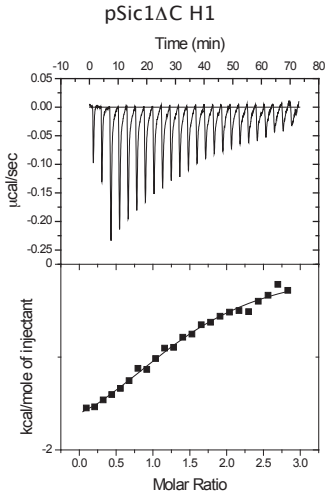
Figure 4.13. ITC with Cks1 and Sic1ΔC Mutant Constructs. Sic1ΔC T33 contains only one phosphorylation site, T33, with all the other S/TP mutated to AA. Sic1ΔC -2P is the same as Sic1ΔC T33 but substitutes a proline in the -2 position of T33, increasing the affinity for Cks1. Sic1ΔC H1 is the same as previous but replaces the sequence context of T33 with that of histone H1 Cdk1 site PK{pT}PKKAKKL to test the effect of downstream lysines on Cks1 binding. Sic1ΔC AB1 is the same as previous but a stretch of residues 48-50 was mutated from STT to AAA, removing the phosphoacceptor S48 for which phosphorylation of T33 primes.

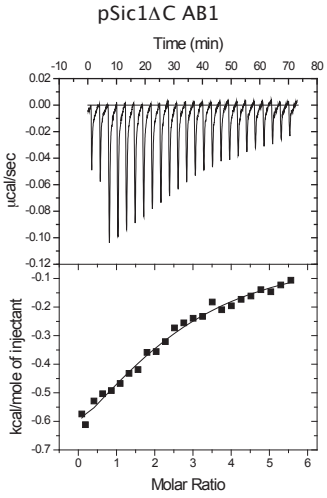

Sic1ΔC
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

pSic1ΔC
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio


pSic1ΔC T33
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

pSic1ΔC -2P
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio


pSic1ΔC H1
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio


pSic1ΔC AB1
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

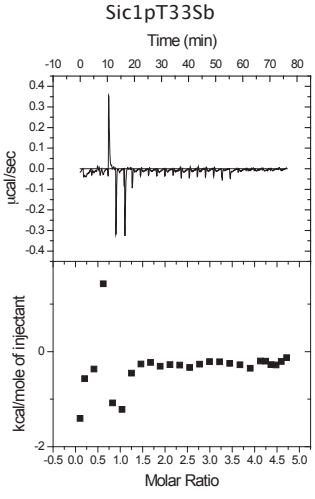
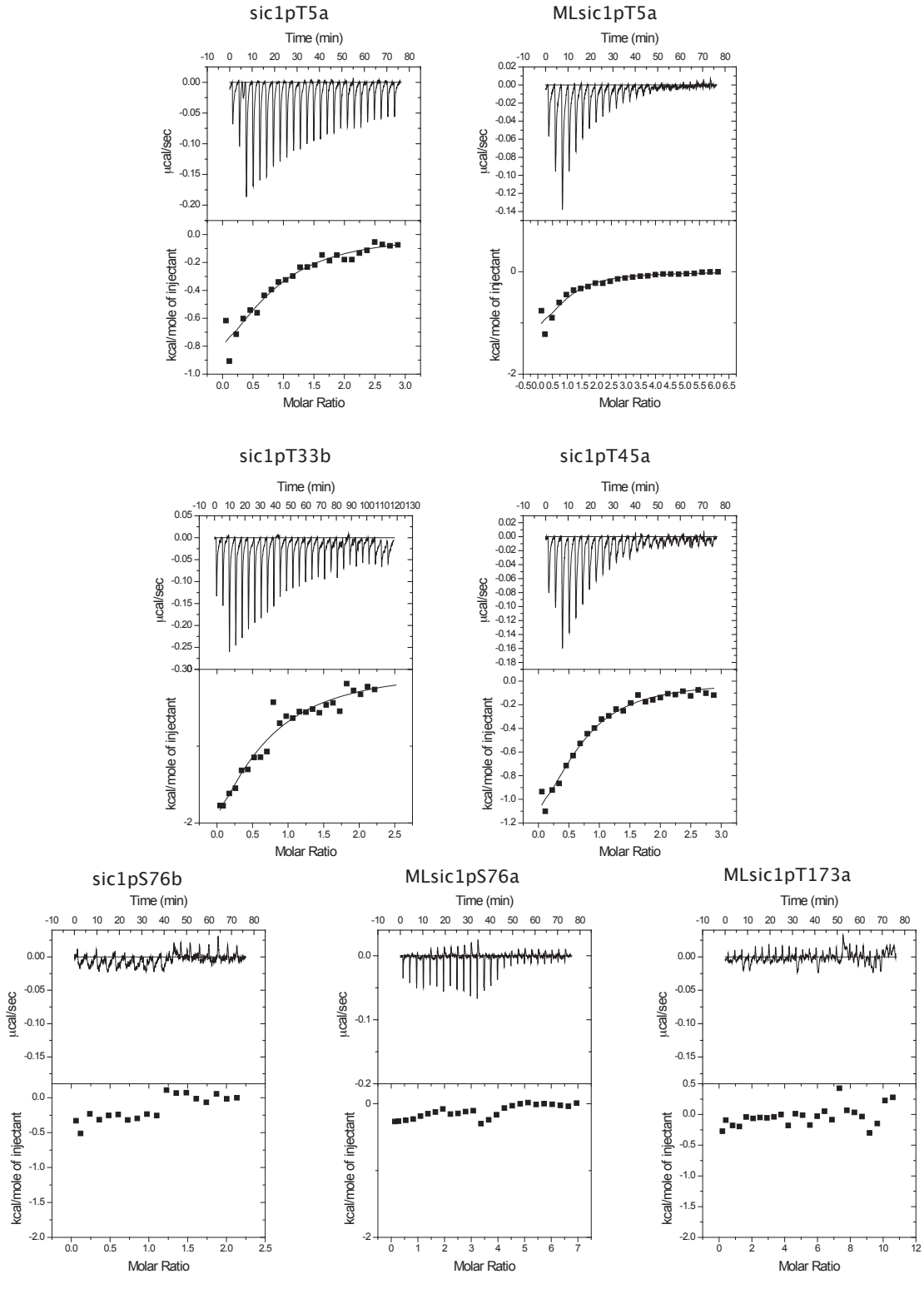

Sic1pT33Sb
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

Figure 4.14. ITC with Cks1 and Sic1ΔC Single Site Constructs. All S/TP sites except the site in the name of the construct are mutated to AA. Some constructs are labeled with the prefix "ML" because they were phosphorylated by the Mart Loog group, while the rest were phosphorylated by me using recombinant human Cdk. S76 and T173 are very poor Cdk sites in both the United States and Estonia.


sic1pT5a
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
MLsic1pT5a
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
sic1pT33b
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
sic1pT45a
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
sic1pS76b
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
MLsic1pS76a
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
MLsic1pT173a
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio

4.3. Discussion

Our investigation of Cks1 and pCdc6 began by questioning the conclusions of Mimura et al. (2004). The Cdc6 NTD contains not only a Cdc4-binding phosphodegron, but also a phosphorylation dependent Cdk-interacting domain (Mimura et al. 2004). In this study, Cdc6 NTD-bound beads were incubated with G2/M yeast extracts and assayed for their ability to pull down Clb2/Cdk1. Cdc6 NTD possessing either phosphorylated sites 1 and 2 or phosphorylated sites 3 and 4 (see Figure 4.4) was sufficient to pull down Clb2-Cdk, while a four-times phosphorylated peptide that consisted of randomized non-Cdc6 sequence was not capable of binding Clb2, indicating that sequence context is important and phosphoSer/Thr is not sufficient for Clb2 binding.

In the supplementary information Mimura et al. (2004) reports experiments designed to show that Cks1 and Cdk1 are dispensible for Cdc6 NTD binding to Clb2. To deplete Cks1 from yeast extracts the authors used a strain in which the *cks1* gene was fused to an N-terminal heat-inducible degron (Sanchez-Diaz et al. 2004). Levels of Cks1 were indeed below those detectable by immunoblot; however that does not exclude the possibility that sufficient Cks1 remains to promote Cdc6/Clb2/Cdk association. The authors also perform Cdc6 NTD pulldowns in a Δ*cks1* background. In both the Cks1-depleted and Δ*cks1* G2/M yeast extracts site-specific phosphorylation of Cdc6 NTD still promotes association with Clb2 and Cdk1. This association is not disrupted by elimination of two predicted RXL Clb2 binding motifs. Comparing Cdk1 binding to Cdc6 NTD

beads from G1/S extracts versus G2/M extracts, Cdk1 from G2/M extracts binds both unphosphorylated and phosphorylated Cdc6 NTD, while G1/S Cdk1 fails to bind Cdc6 NTD.

On the basis of these data the authors propose direct binding between Clb2 and a phosphoCdc6 motif. There are a number of results that are not explained by this conclusion. First, it appears that in Cks1-depleted and Δcks1 pulldowns Cdc6 NTD still pulls down Cdk1 and, to a lesser extent, Clb2 independent of the extent and sequence context of Cdc6 phosphorylation. Curiously, these interactions are not observed the wild-type conditions in either experiment. Second, Cdk1 from G2/M extracts binds unphosphorylated and phosphorylated Cdc6 NTD, suggesting the presence of a Clb2-dependent but phosphorylation-independent association. Third, recent detailed kinetic studies in yeast show that Clb2/Cdk substrate preference is not hydrophobic patch dependent but instead determined by phosphoacceptor site sequence context (Kõivomägi, Valk, Venta, Iofik, Lepiku, Morgan, et al. 2011). This discovery means that the persistence of Cdc6/Clb2/Cdk1 association upon mutation of Cy motifs in Cdc6 is unsurprising. An alternative hypothesis is that Clb2 binding rearranges the active site of Cdk1 to allow formation of a stable complex with phosphoCdc6. The docking-independent specificity profiles conferred upon Cdk1 by different cyclins suggest this is possible. Significant inhibitory ability of Cdc6 toward Clb2/Cdk but not Clb5 or Clb3/Cdk has also been reported, suggesting some kind of direct Clb2-specific interaction, the biological consequences of which

would be relevant to mitotic exit (Kõivomägi, Valk, Venta, Iofik, Lepiku, Morgan, et al. 2011).

Regardless of the complexity of interpreting the results that appear within Mimura et al. (2004), it is important to note that nowhere within this study did the authors attempt to demonstrate a phosphorylation-dependent association between Cdc6 and Clb2 with purified components. There are several obvious experiments that could have performed to test the prediction that phosphoCdc6 NTD directly binds Clb2 and not Cdk1 or Cks1. The work contained within this chapter begins soon after our lab undertook some of these direct binding experiments. In our hands, pull-down experiments with phosphoCdc6 and Clb2 show no phosphorylation-dependent binding and low amounts of binding overall.

Our proteomic approach for identifying a Cks1 phosphopeptide-binding consensus was inspired by a similar strategies used in the identification of Cdc4-binding phosphodegrons (CPD) of SCF ubiquitin ligase substrates (Nash et al. 2001). The Cdc4 CPD motif is L/I-L/I/P-pT-P$[RK]_4$, where $[RK]_4$ indicates that basic residues are disfavored in the +2 to +5 positions following the phosphothreonine. The similarities between the Cks1 consensus and the Cdc4 consensus are striking: preferences for hydrophobic residues in the -2 position, requirement for pThr in the 0 position, requirement for Pro in the +1 position, and disfavored basic residues in the +2 position. So far, Cdc6 and Sic1 have been found to directly associate with Cks1, and correspondence with Mart Loog

suggests that Far1 may also be a Cks1-dependent Cdk substrate. Cdc6, Sic1, and Far1 are also all ubiquitinated in a Cdc4 dependent manner. I predict that we will find considerable overlap in the sets of Cks1-directed CDK substrates and Cdc4-directed SCF substrates, and that there will be regulatory significance to this relationship, specifically in terms of Cks1's role at the G1/S transition. A proper comparison will have to await characterization of Cks1-binding phosphoproteins *in vivo*.

There is obvious utility in the identification of a consensus Cks1-binding phosphopeptide from a crystallographer's standpoint. One of the roadblocks to the co-crystal structure of pCdc6-T7/Cks1 may have been the relatively low affinity of this complex. Heterogeneity in the phosphate-binding pocket could interfere with the formation of crystal contacts, especially because the phosphate-binding pocket is common packing interface in existing Cks1 structures. Even if co-crystals were obtained, incomplete occupancy could reduce the quality of the electron density of the phosphopeptide. An optimal phosphopeptide could minimize these problems. Additionally, identification of phosphopeptide positions that do not contribute to the specificity of the interaction inform the design of crystal contacts engineered to employ the phosphopeptide.

Beyond structural characterization of the Cks1-phosphoprotein interaction at the phosphate-binding site, there are broader outstanding structural questions about the relationship between Cks1 and Cdk specificity.

While the Cdk2/CksHs1 structure shows no conformational differences between free and Cks-bound Cdk2, this conclusion may not be generalizable to yeast Cdks. Mart Loog has observed that Cks1 confers phosphoacceptor site sequence context preference to Cdk1, suggesting that conformational changes take place at or affecting the active site. Work from the Deshaies group also indicates that Cln/Cdks and Clb/Cdks are differently influenced by Cks1, with Cln/Cdks requiring Cks1 for activity. While the number of biologists who would be seriously interested in yeast Cdk(/cyclin/Cks) structures may be limited to our immediate science friends and families, together we are well-equipped to make the most of these investigations, applying findings and testing hypotheses in a variety of experimental systems.

In addition to stimulating Cdk activity toward multiphosphorylated substrates, our research suggests that Cks1 plays an important role in targeting Cdk to its inhibitors. Sic1, Cdc6, Swe1, and Far1 are all Cdk inhibitors, and the Cks-stimulated Cdk substrate Cdc27 also contributes to Cdk inhibition by activating the APC, promoting cyclin degradation. Additionally, Cks1 has also been implicated in activation of the APC^{CDC20}, which targets Clb2 for degradation (Rudner and Murray 2000). This is consistent with a hypothesis that posits a multipurpose role for Cks1 in Cdk inactivation at cell cycle checkpoints and mitotic exit. Over 25 years after it was suggested by genetic experiments, Cks1 still appears to be both a positive and negative regulator of Cdk, both stimulating multisite phosphorylation and targeting the kinase to inhibitors and inactivators.

4.4. Materials and Methods

4.4.1. Protein Expression and Purification

Three different Cks1 constructs were used in this study: GST-Cks1, His_6-Cks1, and tagless Cks1. GST-Cks1 was expressed from a pGEX vector with a TEV protease site and purified by gluthatione sepharose chromatography. His_6-Cks1 was expressed from a pAL vector with chloramphenicol resistance and a thrombin protease site, although the tag was never removed. His_6-Cks1 was purified using Ni-NTA chromatography followed by size exclusion chromatography using a Superdex 75 column in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT. Tagless Cks1 was expressed from a modified pET vector and purified using DEAE cation exchange chromatography followed by Source 15Q anion exchange chromatography and size exclusion as described for His_6-Cks1. All Cks1 constructs were expressed in *E. coli* BLD1(DE3) competent cells induced by 1 mM IPTG overnight at 22°C.

Natively unfolded His_6-Cdc6 NTD (residues 1-48) and His_6-Swe1 NTD (residues 1-425) constructs were expressed from pAL vectors with chloramphenicol resistance and a thrombin cut site. To prevent degradation, expression was induced by 1 mM IPTG for 2-4 hours at 37°C and constructs were purified over Ni-NTA resin under denaturing conditions (6M urea, 200 mM NaCl). His_6-Cdc6 NTD was eluted into native buffer conditions plus 400 mM imidazole, while His_6-Swe1 NTD was eluted in denaturing buffer +400 mM imidazole and further purified by C4 HPLC reverse phase chromatography. His_6-

Swe1 NTD was then lyophilized and resuspended in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT. These methods were adopted because Swe1 degradation was otherwise difficult to prevent. His_6-Swe1 NTD-N (residues 1-212) may be expressed and purified in the same manner, although degradation is less problematic and expression is improved, so one may choose to alter the purification strategy to return the protein to native conditions without lyophilization and HPLC. A His_6-Swe1 NTD-C (residues 212-425) construct was too unstable to be experimentally useful.

4.4.2. Isothermal Titration Calorimetry

Sic1ΔC constructs for calorimetry were received from Mart Loog shipped on dry ice. On the occasions where the ice was gone, the samples remained stable and no precipitation was observed. The Loog group typically sends the proteins in glycerol, so they may be immediately frozen upon receipt. Sic1ΔC and Cks1 were dialyzed together overnight in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, and, for many experiments, 1 mM DTT. The presence of DTT created erratic baseline problems. In the process of trying to identify the source of these baseline problems, Cks1 P93A was substituted for wild-type Cks1 and, for the record, also binds phosphorylated Sic1ΔC. However, all data provided for Mart Loog were obtained using wild-type Cks1. While phosphorylated Sic1ΔC binds Cks1 with only moderate affinity, the heat of binding is quite large. Therefore to allow saturation, optimal Sic1ΔC concentration for ITC is only 20-30 μM. Optimal Cks1 concentration is ~ 0.5 mM.

4.4.3. Spot Blots

Lyophilized phosphopeptides were resuspended in sterile PBS pH 7.4. Resuspended peptides were mixed with 1 mg/ml BSA resuspended in PBS pH 7.4 for a final concentration of 1.8 mM phosphopeptide. An equal volume of 0.2% glutaraldehyde was added and the mixture was spun for 60 minutes at room temperature. The cross-linking reaction was quenched by stirring with an equal volume of 1M glycine for 30 minutes at room temperature. BSA-conjugated phosphopeptides were dialyzed against TBS pH 7.4 for 3 hours, replacing dialysis buffer each hour. Cross-linked peptides were spotted (typically 0.5-3 µl) directly onto an activated PVDF membrane. A detailed protocol is provided in the Appendix.

4.4.5. Synthetic Arrays

His_6-Cks1 was alkylated using a three step protocol: (1) addition of 10 mM DTT; mix and set 10 minutes on ice; (2) addition of 25 mM iodoacetic acid (IAA); mix and set 30 minutes room temperature in the dark; (3) quench with 20 mM DTT. One milliliter stocks of 1M IAA and DTT were always made fresh. Peptide arrays were synthesized on amino-PEG cellulose membranes by the MIT Biopolymers Laboratory. Briefly, arrays were blocked for one hour then incubated overnight at 4°C with 36 µg/ml His_6-Cks1-al. Membranes were probed for two hours at room temperature with 1:2000 anti-His_6-HRP antibody (SCBT) and developed using SuperSignal West Dura ECL reagents. A detailed protocol is provided in the Appendix.

4.5 References

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APPENDIX

A1. Spot Blots with BSA-Conjugated Peptides

Materials

Protocol 1: Coupling Peptides to Bovine Serum Albumin

1. Phosphopeptides, lyophilized (22 total, 4 mg in each tube)
2. BSA (1 ml 10 mg/ml in 1XPBS)
3. 10X PBS (recipe 1)
4. 10X TBS (recipe 2)
5. 0.2% Glutaraldehyde (996 µl H2O + 4 µl 50% glutaraldehyde stock)
6. 1M glycine

Protocol 2: Blocking, Probing, and Detecting

1. Peptide samples from Protocol 1
2. PVDF membrane or nitrocellulose, cut to appropriate size and positions marked gently with pencil or VWR marker (not Sharpie)
3. Incubation buffer (recipe 3)
4. TBS (recipe 2)
5. Blocking buffer (recipe 4)
6. His-Cks1, ideally ~5 mg/ml or more.
7. SCBT anti-His-HRP antibody

8. Various sizes of boxes for different size blots (use the smallest you can to minimize antibody use but that still allows the blot to float freely and be covered by buffer at all times)
9. Saran wrap
10. Film or Bio-Rad Gel Doc darkroom machine (Partch Lab)

Methods

Recipe 1: 1L 10X PBS

Dissolve the following in 800 ml distilled H_2O.

80g of NaCl

2.0g of KCl

14.4g of Na_2HPO_4

2.4g of KH_2PO_4

Adjust pH to 7.4.

Adjust volume to 1L with additional distilled H_2O.

Sterilize by autoclaving.

Recipe 2: 1L 10X TBS

250 mM Tris.HCl = 39.4g

1500 mM NaCl = 87.66g

Adjust pH to 7.4.

Adjust volume to 1L with additional distilled H_2O

Sterilize by autoclaving.

Recipe 3: Incubation Buffer (make fresh)

20 mM Tris HCl pH 7.4

0.5 M NaCl

3% w/v milk powder

Recipe 4: Blocking Buffer (make fresh)

TBS

5% w/v milk powder

0.2% Triton X-100

Protocol 1: Coupling Peptides to Bovine Serum Albumin

1. Dissolve BSA and the peptide in PBS at pH 7.4 to a final concentration of 1 mg/ml BSA and 1.8 mM peptide (original protocol recommends 300-600 μM)
2. Add dropwise and slowly (to prevent precipitation) an equal volume of 0.2% glutaraldehyde under constant stirring at room temperature. Glutaraldehyde cross-links primary amino groups on the peptide to those on the carrier.
3. Stop the reaction after 1 hour by the addition of an equal volume of 1M glycine. Stir the mixture for 30 minutes.
4. Dialyze extensively against TBS (3 x 1 hour).

Protocol 2: Blocking, Probing, and Detecting

1. Spot peptide samples onto membrane. Start with 0.75-2.5 μl. If using PVDF, activate the membrane first with MeOH.

2. Incubate membrane for 4 hours at room temperature (or, for better blocking, overnight at 4°C) with Incubation Buffer to block the free protein binding sites.
3. Incubate the membrane for 2 hours at room temperature (or overnight at 4°C) in Blocking Buffer and 36 µg/ml (2 µM) His-Cks1 (original protocol recommends as low as 2.5 µg/ml). As a negative control, another blot can be incubated in parallel without Cks1.
4. Wash the blots 3x10 minutes with Blocking Buffer. Incubate the blots 1-2 hours at room temperature with anti-His-HRP at 1:2000 in Blocking Buffer.
5. Wash the blots once with Blocking Buffer and twice with TBS for 10 minutes and develop the blot by enhanced chemiluminescence (ECL) detection using Supersignal West Dura reagents.

General tips:

- I try to conjugate peptides and spot them in the same day or within a few days if possible. Fresh, unfrozen peptide stocks seem to work best and can be stored at 4°C for a week or more.
- I always make my 0.2% glutaraldehyde and BSA stocks fresh.

- Your limiting material in peptide conjugation will probably be tiny stir bars. Take inventory. The 96-well plates hold 0.5 ml tubes nicely.
- Tiny dialysis vessels can be constructed from the tops of eppendorf tubes. Use a razor blade to cut the bottoms off at about the 1 ml mark. Cut small squares of dialysis tubing and practice making a good seal and not losing your sample. Recovering sample is easiest if rather than opening the cap, you puncture the dialysis membrane with the 100 µl glass syringe and suck out your sample. Make sure to completely pour or shake off the dialysis buffer on top of the membrane before puncturing or your sample will be diluted.
- To prevent smudges, avoid touching membranes with ungloved hands at all times. Handle them by the edges or, preferably, with tweezers.
- Do not leave PVDF in MeOH for too long or it will inactivate. Do not put nitrocellulose in MeOH at all or it will dissolve completely.
- Make sure your milk solutions are totally dissolved—chunks will not block well and might make your blots look grainy.

- After applying SuperSignal, make sure to sop up the excess to reduce background using a clean kimwipe around the edges of your blot.
- The darkroom is in METOX and there is a reservation sheet for the developer. The contact person for training is Karen Ottemann.
- An initial 10 second exposure is a good place to start.

A2. Synthetic Peptide Arrays

Materials:

Protocol 3: Probing, Blotting, and Detecting

1. Positional Scanning Array NOT from Genscript
2. Incubation Buffer (recipe 3)
3. TBS (recipe 2)
4. Blocking Buffer (recipe 4)
5. His-Cks1-al, ideally ~5 mg/ml or more.
6. SCBT anti-His-HRP antibody

Protocol 4:

1. Stripping Buffer A (recipe 5)
2. Stripping buffer B (recipe 6)
3. Sonicating bath
4. TBS-T (recipe 7)
5. Ziploc bags

Methods

Recipe 5: 200 ml Stripping Buffer A

8M urea

1% SDS

PBS

Add 0.5% β-ME fresh and adjust to pH 7.0 with acetic acid.

Recipe 6: 200 ml Stripping Buffer B

10% acetic acid

50% ethanol

40% H_2O

Recipe 7: 500 ml TBS/T

TBS

0.05% Tween-20

Protocol 3: Probing the Positional Scanning Array

1. Block membrane for 1 hr at room temperature with Incubation Buffer to block the free protein binding sites.
2. Incubate the membrane overnight at 4°C in Blocking Buffer and 36 µg/ml (2 µM) His-Cks1.
3. Wash the blots 3x10 minutes with Blocking Buffer. Incubate the blots 2 hours at room temperature with anti-His-HRP at 1:2000 in Blocking Buffer.

4. Wash the blots once with blocking buffer and twice with TBS for 10 min and develop the blot by enhanced chemiluminescence (ECL) detection using SuperSignal West Dura reagents.

Protocol 4: Stripping the Membrane [adapted from (Frank & Dubel, 2006)]

1. Wash membranes 2 x 10 minutes in H_2O
2. Place membranes in a ziploc bag with Stripping Buffer A and sonciate in bath sonicator 3 x 10 minutes at 40°C, replacing the buffer each time.
3. Repeat Step 2 with Stripping Buffer B.
4. Wash membranes 3 x 10 minutes with H_2O for immediate use, or 3 x 10 minutes alcohol if drying to store. Store dry membranes at -20°C.

General Tips:

- Nickel could potentially be oxidizing to the peptides on the membrane. Make sure there is no leached nickel remaining from His-Cks1 purification by chelating with EDTA, dialyzing, or running ion exchange.
- Azide is toxic—wear gloves.
- Stripping Buffer A has a very unpleasant smell because of the β-ME. Consider working in the hood.

- Before reprobing, wash 2x10 minutes in plenty of TBS to get rid of azide, which can inhibit HRP.
- See truncation array tips above.

A3. References

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