10-28-2010 12:00 AM

Mitotic Regulation of Protein Kinase CK2

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Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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(Format: Integrated Article)

by

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A Thesis submitted in partial fulfillment for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada
November, 2010

The University of Western Ontario
The School of Graduate and Postdoctoral Studies

Certificate of Examination

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The thesis by
Nicole St-Denis
entitled:

Mitotic Regulation of Protein Kinase CK2

is accepted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Date

Chair of Examining Board

Abstract

Protein kinase CK2 is a serine/threonine kinase with a multitude of substrates and roles in many cellular processes, including mitosis. CK2 is constitutively active, yet we hypothesize that CK2 is indeed regulated in mitosis through subtle means, enabling CK2 to perform its functions unique to cell division. Our aims were to examine the roles of mitotic phosphorylation, subcellular localization, and interplay with mitotic kinases in the regulation of CK2 activity.

We first examined the role of four highly conserved mitotic phosphorylation sites located in the unique C-terminus of CK2α. Phosphospecific antibodies generated against the sites show that CK2α phosphorylation is temporally regulated and occurs during prophase and metaphase during normal mitotic progression. Proper phosphorylation of CK2α is required for proper mitotic progression, as stable cell lines expressing phosphorylation site mutants of CK2α display severe mitotic defects.

We next examined the impact of these phosphorylation events on the subcellular localization of CK2. We show that CK2α, but not CK2α', localizes to the mitotic spindle. Localization of CK2α to the mitotic spindle is phosphodependent, and requires the peptidyl-prolyl isomerase Pin1. These results are a rare example of functional divergence between the two catalytic isoforms of CK2, and suggest that the role of CK2α phosphorylation during mitosis is to promote localization of CK2 to the mitotic spindle.

Finally, we examined the possibility that CK2 activity during mitosis is regulated through hierarchal phosphorylation events, wherein CK2 would phosphorylate proteins only after priming phosphorylation events catalyzed by other mitotic kinases, particularly Cdk1. As this phenomenon has never been systematically investigated, we have

investigated the consensus requirements for CK2 primed phosphorylation, and in particular Cdk/CK2 hierarchical phosphorylation. A genome-wide search for potential mitotic substrates matching the consensus sequence suggests that Cdk1/CK2 hierarchical phosphorylation may indeed contribute to mitotic signaling, particularly on the mitotic spindle.

Taken together, our results confirm the importance of CK2 in mitotic cell division, and highlight several examples of subtle regulation of CK2, through phosphorylation, subcellular localization, and interplay with other protein kinases. This helps explain how CK2, a constitutively active kinase, can participate in tightly regulated cellular processes like mitosis.

Keywords: Protein kinase CK2, mitosis, mitotic spindle, phosphorylation, localization, Pin1, hierarchical phosphorylation, Cdk1, spindle assembly checkpoint.

Coauthorship

Chapter 2:

Rich Derksen generated the stable cell lines expressing CK2α phosphorylation mutants and CK2β used in these studies, and performed the kinase assays in Figure 2.4D and the proliferation experiments shown in Figure 2.5.

Chapter 3:

Erin Parker generated the stable cell lines expressing Pin1 and Luciferase shRNAs used in these studies, and Melanie Bailey generated the Flag-Pin1 plasmids.

Chapter 4:

Soluble peptides were generated by Huadong Liu in the laboratory of Dr. Shawn Li, and the peptide match program used to search the genome was written by Dr. Greg Gloor.

Acknowledgements

First and foremost, thanks to Dr. Dave Litchfield. Thanks for all your advice, guidance, support, constructive criticism, funny stories about random people in science, smooth phrasings for potentially awkward correspondence, clever analogies, and jokes about both my lack of a Mac and the ubiquity of my iPod. Thanks for dealing with all my tears, rants, happy dances, and everything in between. The post-it notes were funny, but seriously, you really are my hero.

I would also like to thank everyone at UWO that took the time to help this project progress. My advisory committee, Dr. Eric Ball and Dr. Fred Dick, were a great source of guidance and advice. Thanks also to Drs. Shawn Li, Greg Gloor, Lina Dagnino, Marc Tini, and David Rodenhiser for their contributions to this project and my development as a scientist. A special thanks to Dr. Rob Hegele, who gave me my first real science job when I was just a little undergrad, and through his infectious enthusiasm, helped me fall in love with science. The flow cytometry performed in Chapter 2 would never have happened without the help of Dr. Kristin Chadwick at the London Regional Flow Cytometry Facility. Thanks also to Dr. Dale Laird for the use of his confocal, and Jamie Simek for his guidance in how to actually go about using the confocal.

During my time in the Litchfield lab, I have had the opportunity to work with a wide variety of people, all of whom were that special kind of insane that compels people to mix stuff they can't see in tubes all day. Without all of these people, the last five years of my life would have been much less entertaining**,** and I'm grateful to each of them for their friendship, support, and unique perspectives on life and science: Melanie Bailey, Dave Canton, Victoria Clarke, Rich Derksen, James Duncan, Kelly Duncan, Ashley

French, Michelle Gabriel, Kathryn Garside, Laszlo Gyenis, Angela Holmes, Kristina Jurcic, Mary Ellen Olsten, Dana Onica, Erin Parker, Jennifer Raaf, Jake Turowec, Greg Vilk, Kelly Weedmark, and Cunjie Zhang. Special thanks go out to my person, Erin Parker; the founding co-president of Club Pure Awesome, Jake Turowec; Angela Holmes and Kathryn Garside for being two of my favourite people ever; Ashley French for being the world's greatest lab mom; the neighbour kids, Ryan Mohan and Anita Rao, who were always ready for beer and sushi ("Okay, I just have to split my cells"); and everyone that made the 'boy's office' such a good time.

Thanks also to all of my friends, UWO-related and otherwise, for being unfailingly supportive even when I had no time for you, for being ridiculously fun when I did, and for being available for beer and distraction when the science was cruel. I am also grateful to my family for their support and understanding over the past five years. The amount of people who have very little understanding of what I do all day yet still brag about me to their friends absolutely staggers me, and I love you for it.

Saving the best for last, I would also like to thank Ryan Ruttgaizer for his love and support throughout this process. Ryan is always prepared to celebrate successes, large or small, but more importantly he's the greatest cheerleader I have, and his support during the tough times was instrumental to the completion of my doctoral work. Now on to the next adventure!

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List of Abbreviations, Symbols and Nomenclature

°C	degrees Celcius
μCi	microCurie
μg	microgram
μl	microliter
μM	micromolar
APC/C	anaphase-promoting complex/cyclosome
ATP	adenosine triphosphate
bp	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAK	Cdk activating kinase
Cdk	cyclin-dependent kinase
CENP-E	centromeric protein E
CK1	Protein Kinase CK1; formerly casein kinase I
CK2	Protein Kinase CK2; formerly casein kinase II
CKIP1	CK2 interacting protein 1 (PLECHO1)
DAG	donkey anti-goat
DAPI	4’,6-diamidino-2-phenylindole
DAR	donkey anti-rabbit
ddH_2O	double distilled water
DMEM	Dulbecco’s modified Eagle Media

DMSO	dimethylsulfoxide
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FACT	facilitating chromatin-mediated transcription
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
FLAG	DYKDDDDK epitope
g	relative centrifugal force
G1	gap 1
G2	gap 2
GAM	goat anti-mouse
GAR	goat anti-rabbit
GFP	green fluorescent protein
GSK3	glycogen synthase kinase 3
GST	glutathione-s transferase
GTP	guanosine triphosphate
HA	hemagglutinin (YPYDVPDY) epitope
HeLa	human cervical carcinoma cells
HRP	horseradish peroxidase
IB	immunoblot

IP	immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactoside
kD	kilodalton
KD	kinase dead
M	mitosis
MAP	microtubule-associated protein
MAPK	mitogen activated protein kinase
MCC	mitotic checkpoint complex
ml	milliliter
mM	millimolar
MMTV	mouse mammary tumor virus
MPM-2	mitotic protein monoclonal-2
MS	mass spectrometry
MSCV	murine stem cell virus
Myc	MASMEQKLISEEDLNN epitope
Na_2VO_4	sodium orthovanadate
NP-40	Nonidet P-40
OA	Okadaic acid
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PBD	polo-box domain
pS	phosphorylated serine residue

pT	phosphorylated threonine residue
pY	phosphorylated tyrosine residue
PCR	polymerase chain reaction
PI	propidium iodide
Pin1	protein interacting with NIMA-1
Plk1	polo-like kinase 1
PMSF	phenylmethylsulfonylfluoride
PP2A	protein phosphatase 2A
PPIase	peptidyl-prolyl isomerase
PVDF	polyvinylidene difluoride
RNAi	RNA interference
rpm	revolutions per minute
S	synthesis
SAC	spindle-assembly checkpoint
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	short interfering RNA
shRNA	short hairpin RNA
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.5% Tween-20
Tet	Tetracycline
TopoII	topoisomerase II
U2OS	Human osteosarcoma cells
V, A	Volts, Amps

Chapter 1: Introduction[1]

1.1 General Introduction

Protein phosphorylation has long been recognized as an important post-translational modification regulating cellular processes (54). In the human genome, 518 distinct protein kinases have been identified (57), and one third of cellular proteins appear to be phosphorylated (1), often at several distinct sites (20). Proper regulation of phosphorylation events is crucial to the proper function of cellular signalling pathways, and loss of regulation of pathways controlling proliferation and survival can lead to cancer (47). Consequently, there is an enormous interest in the development of novel therapeutics targeting kinases and phosphatases (52). Among promising therapeutic targets is the protein kinase CK2 (95), a serine/threonine kinase that is overexpressed in multiple forms of cancer and has oncogenic properties in mice and cultured fibroblasts (69). CK2 has various and often interconnected roles in multiple cellular pathways, however its long list of substrates and subtle means of regulation have made a thorough understanding of CK2 function elusive, even after over 50 years of intensive research.

1.2 The CK2 Family

Originally discovered in 1954 (18), CK2 is a family of enzymes that in humans consists of two catalytic subunits, termed CK2α and CK2α', and one regulatory subunit, CK2β (Figure 1.1). The catalytic subunits show high sequence similarity throughout most of their coding regions, despite the fact that they are transcribed from different genes,

[1] Some of the content in this chapter has been published: St-Denis NA, Litchfield DW. **From Birth to Death: The Role of Protein Kinase CK2 in the Regulation of Cell Proliferation and Survival** (2009). Cellular and Molecular Life Sciences 66:1817-1829.

Figure 1.1

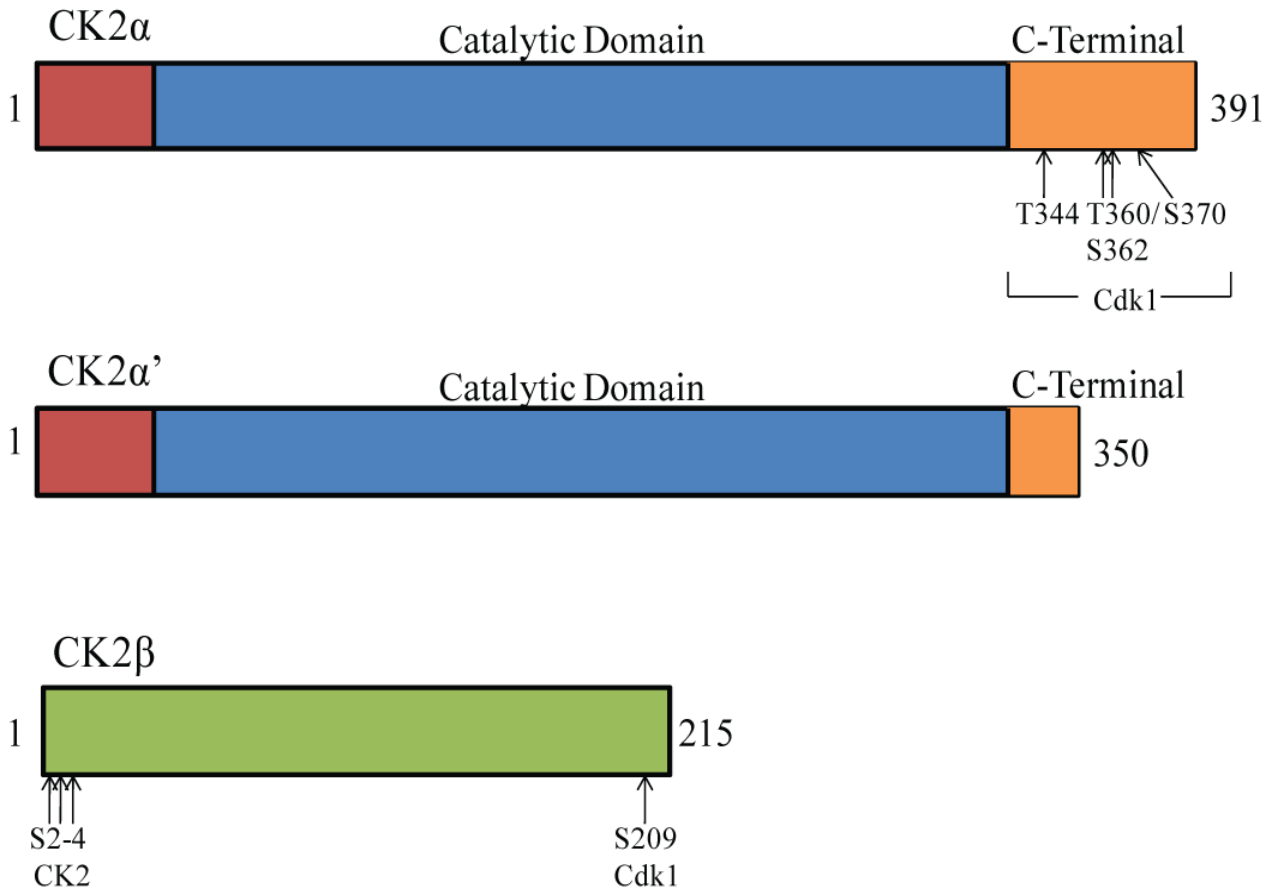

Figure 1.1 Schematic representation of CK2 subunits. Linear representation of the sequences of the catalytic isoforms CK2α and CK2α' and the regulatory subunit CK2β. *In vivo* phosphorylation sites are indicated, including the four mitotic phosphorylation sites on the extended C-terminus of CK2α, and the S209 mitotic phosphorylation site and autophosphorylation sites on CK2β.

CK2α on chromosome 20 (123) and CK2α' on chromosome 16 (125). The catalytic domains of CK2α and CK2α' are approximately 90% identical (71), but have dramatically different C termini, with an extended C-terminal tail on CK2α not present on CK2α' (77). The catalytic subunits are members of the CMGC family of kinases (79), which also includes cyclin-dependent kinases (Cdks), the mitogen-activated protein kinases (MAPKs and ERKs), and glycogen synthase kinase-3 (GSK3). In contrast, CK2β has no extensive homology to any other known proteins (55), making its role difficult to decipher. CK2 subunits are typically found in mammalian cells as a tetramer, in which two CK2β subunits dimerize and two catalytic subunits (α/α, α'/α', or α/α') bind to the CK2β dimer to form a tetramer (35, 40, 42, 90) (Figure 1.2). CK2 has long been distinguished from other serine/threonine kinases by its ability to phosphorylate serine and threonine residues proximal to acidic amino acids (98), as well as by its unique ability to use either ATP or GTP as a phosphate donor (4). Through systematic study of CK2 substrates, Pinna *et al.* have defined the minimal consensus sequence of CK2 phosphorylation as S/T-X-X-Acidic, where X is any amino acid and the acidic residue is typically aspartic acid or glutamic acid (99). Interestingly, phosphorylated serine can also act as the proximal acidic residue, enabling CK2 to participate in sequential phosphorylation events by using residues phosphorylated by itself or other kinases as part of its consensus requirements (2, 99, 121). Though the majority of CK2 substrates conform to the established consensus sequence, there are some exceptions. For example, the serine 392 site in the tumour suppressor protein p53 is phosphorylated by CK2, despite the fact that the sequence does not conform to the CK2 consensus sequence (83). Conversely, the presence of the minimal consensus sequence does not necessarily

indicate that CK2 will efficiently phosphorylate the site *in vivo*, as there may be other determinants present in the amino acid sequence (84). Additionally, phosphorylation of a specific protein at a specific site can be influenced by the many subtle regulatory forces acting on CK2 in mammalian cells.

1.3 Regulation of CK2 Activity

CK2 is ubiquitously expressed in a variety of cell types and tissues, and is considered to be constitutively active, as it is not subject to the strict on/off regulation of other kinases, such as MAPKs and CDKs. However, there exist several subtle mechanisms by which CK2 activity can be focused on one or more substrates while excluding other substrates, and these forms of regulation have been an area of intense study in the field.

1.3.1 Localization

Original work investigating the localization of CK2 involved subcellular fractionation studies; however this line of investigation yielded little insight into the subcellular localization of CK2, as it appeared to be ubiquitously expressed throughout the cell. Development of antibodies against the holoenzyme offered some additional insight, but many advances in the study of CK2 localization came with the development of subunit-specific antibodies, allowing comparison and confirmation of CK2 subunit localization. Polyclonal serum against purified CK2 have been used to show localization to the nucleolus (23) and the plasma membrane (26). Localization at the plasma membrane was later confirmed for CK2α with an antibody targeted against that subunit

Figure 1.2

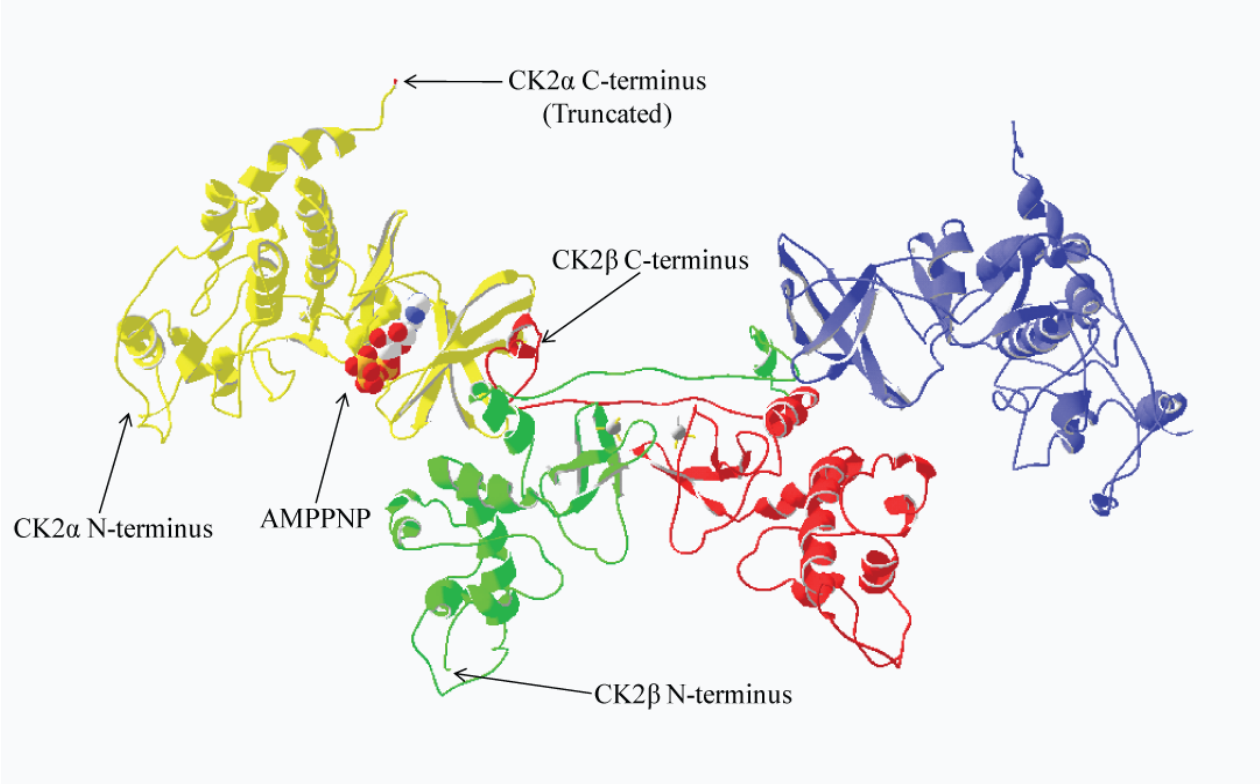

Figure 1.2 Crystal structure of tetrameric CK2. Catalytic subunits are shown in blue and yellow and regulatory subunits are shown in green and red. Also indicated is a non-hydrolyzable ATP analog, AMPPNP, in the active site of one of the catalytic subunits. Note that the C-terminal tail of CK2α is truncated in this structure (missing amino acids 338-391). The structure of the C-terminus is unknown. This representation of the CK2 tetramer was generated using Swiss PDB Viewer; PDB File 1JWH.

(105). Through the use of subunit-specific antibodies and identification of organelle-specific substrates, CK2 subunits have been localized in the cytoplasm (31, 127) and nucleus (62, 105), and colocalizing with the cytoskeleton (31), centrosomes (62, 82), mitotic spindle (62, 127), mitochondria (103), endoplasmic reticulum (94), nuclear matrix (114, 115), nucleolus (8, 97), and nucleosomes (43). Surprisingly, there even exists a CK2-like ectokinase present on the surface of cells (100, 120). Despite all this work, there remains some ambiguity as to the precise localization of CK2, as these studies were conducted using a variety of techniques, antibodies, fixation methods, and cell lines. This also explains the sometimes conflicting results reported in the literature.

As mentioned above, the development of subunit specific antibodies has allowed study of the localization of individual subunits both in tetrameric form and as individual protein entities. This has lead to extended knowledge of CK2 localization patterns, but has also introduced a new level of complexity to the study of CK2 regulation. Expression of fluorescently-tagged versions of CK2α and CK2β have shown differences in localization, and also show differences in how efficiently they can dynamically shuttle between the nucleus and the cytoplasm (32). Since the binding affinity between CK2α and CK2β is high (10), this indicates the presence of discrete subpopulations of free catalytic subunits, with specialized functions from the tetramer.

Clearly, the localization of CK2 is dynamic, and seems to depend on a cell's requirements for CK2 activity in space and time. Often, specific localization of CK2 depends on protein-protein interactions with a variety of cellular proteins, and often leads to phosphorylation of specific substrates in those locations.

1.3.2 Protein-Protein Interactions

CK2 localization is an important mechanism in targeting the kinase to its proper substrates at any given time, and this localization is largely mediated through protein-protein interactions. These interactions can be subdivided into two types: interactions between CK2 family members, and interactions between CK2 and other cellular proteins. While CK2 is normally found in tetrameric form, it is unknown whether tetramers of different composition have different roles in the cells, but this may represent another level of CK2 regulation. CK2β, termed a 'regulatory' subunit due to the resemblance of the CK2 tetramer to other tetrameric kinases such as PKA, does not actually confer much regulation on the activity of the tetramer, however there are some substrates that are more efficiently phosphorylated by individual catalytic subunits in the absence of CK2β (81). In fact, CK2β has been demonstrated to have several signalling functions in the cell that are completely unrelated to the catalytic activity of CK2 (Reviewed in (10)). In 2002, Martel *et al.* investigated the role of CK2β in the phosphorylation of CK2 substrates, and scored CK2 substrates into three classes, based on the impact of CK2β on the efficiency of phosphorylation (81). The first substrate class included substrates that show a strict requirement for CK2β for phosphorylation, including p53, Topoisomerase IIα, and Cdc25B. Class II substrates, such as Nucleolin and Hsp90, are unaffected by the presence or absence of CK2β, while class III substrates like Calmodulin and S100B are phosphorylated only in the absence of CK2β (81). This research showed that tetrameric composition of CK2 can be modulated for different substrates. An interesting possibility concerns the formation of high molecular weight structures consisting of large numbers

of CK2 tetramers. CK2 tetramers can self-aggregate *in vitro*, and reproducibly and reversibly form organized higher structures like 4-tetramer rings and larger, filamentous structures (117). It is unknown whether these higher order structures are present *in vivo*, but if so, they would further expand the sophistication of CK2 regulation through subunit composition.

Extending past CK2 subunit composition, CK2 can also be regulated through interactions with other cellular proteins. Localization of CK2 to various subcellular locales, for example, seems to be largely mediated by protein-protein interactions. These interacting proteins include β-Tubulin (31) and CKIP-1 (now termed PlecHo1) (15), which target CK2 to the microtubules and plasma membrane respectively. There are also several proteins that seem to directly alter CK2 catalytic activity or stabilize the enzyme purely through binding, such as FGF-1 and -2 (14, 109), Hsp90 (86), and Cdc37 (61). Additionally, several interactors seem to reflect enzyme-substrate interactions, such as nucleolin (65), Topoisomerase IIα (12) and Nopp140 (66). There are also interacting proteins that, though binding to CK2, can modulate its catalytic activity to specific substrates. Interactions between CK2 and Pin1, a peptidyl-prolyl isomerase, lead to inhibition of Topoisomerase II phosphorylation (85). Conversely, interaction between CK2 and the FACT complex (composed of hSpt16 and SSRP1), which occurs in response to UV light, leads to enhanced phosphorylation of p53 (60). While it seems that interactions with other cellular proteins can have varying effects on CK2, it is clear that protein-protein interactions do play a role in regulating the localization, activity, and substrate specificity of CK2.

Several CK2 interacting proteins mediate their interactions through binding with CK2β, including some mentioned above (12, 14, 66). Additionally, the CK2β subunit has several additional interacting proteins unique from the catalytic subunits, including the protein kinases c-Mos (19), A-Raf (13, 46), and Chk1 (41), which suggests that CK2β acts as a promiscuous interacting subunit for various kinases (10). While most CK2 interacting proteins show little specificity between the two CK2 catalytic subunits, there are a few substrates and interacting proteins which specifically interact with either CK2α or CK2α'. For example, CK2α, but not CK2α', binds to the serine/threonine phosphatase PP2A (49), CKIP-1 (15), and Pin1 (85) in mammalian cells. Several large scale endeavours have been undertaken to identify and catalogue CK2 interactors in yeast, using two-hybrid assays and large proteomic interaction screens (21, 34, 51, 64). These screens have highlighted the many varied functions for CK2 in yeast, displaying a large amount of interacting partners. Also, comparison between different CK2 subunits shows that at least some of these interacting proteins show functional specialization for one or more CK2 subunits. Large scale interaction studies in mammalian systems are much less comprehensive, due to the increased technical challenges of isolating and identifying protein complexes from mammalian cells (29, 102). However, these global screens seem to confirm that the large numbers of interaction partners and the functional specialization between subunits observed in yeast are also present in mammalian systems (45). Clearly, much needs to be accomplished before we can achieve a validated "CK2 interactome" and can use this catalogue of CK2 interacting proteins to elucidate the roles and regulatory patterns of CK2 activity.

1.3.3 Phosphorylation

Another mechanism governing the subtle regulation of CK2 is phosphorylation of its subunits. CK2β is autophosphorylated following tetramer assembly at serines 2, 3 and 4 (70). Mutation of the sites increases the stability of CK2β, suggesting that autophosphorylation may regulate ubiquitination and proteasome-dependent degradation of CK2β in cells, essentially regulating the levels of protein (128). In addition to the autophosphorylation sites, CK2β is also phosphorylated in a cell cycle dependent manner by Cdk1 at serine 209 (70). CK2β can also be phosphorylated *in vitro* by Chk1 at T213 (63). The purpose of CK2β phosphorylation remains unknown. The CK2α catalytic subunit is also phosphorylated in a cell cycle dependent manner by Cdk1, at four residues in its unique C-terminus (threonines 344 and 360 and serines 362 and 370) (17, 72). The presence of these sites indicates independent regulation of the CK2 catalytic subunits in the cell cycle, and a specialized role for CK2α (or α2β2 tetrameric forms) in mitosis. These phosphorylation sites do not directly affect CK2 activity (16), but it is hypothesized that they may contribute to subtle regulation of CK2, by forming binding sites for interacting proteins and/or targeting CK2 towards favourable substrates or away from unfavourable substrates in the context of mitotic progression.

Many protein kinases, including MAPKs, require phosphorylation on the activation loops present in their catalytic sites for maximal catalytic activity, either through autophosphorylation or phosphorylation by upstream kinases in a signal transduction pathway (56). While no *in vivo* evidence exists to indicate that CK2 activity is increased by activation loop phosphorylation, CK2 catalytic subunits have been shown

to be tyrosine phosphorylated. Donella-Deana *et al.* showed that recombinant catalytic subunits of CK2 are autophosphorylated at tyrosine 182 in the CK2 activation loop in an intermolecular (trans) fashion (25). This was not the first indication that CK2 may act as a dual-specificity kinase, as previous reports had shown CK2 phosphorylation of the nucleolar immunophilin Fpr3 at tyrosine 184 both in yeast and *in vitro*, albeit with less favourable enzyme kinetics than with serine phosphorylation (80, 122). Recent work in our laboratory has confirmed that tyrosine phosphorylation can indeed be catalyzed by CK2 *in vivo*. Phosphorylation of CK2 at tyrosine 182 is dependent on CK2 activity and decreases in the presence of CK2 inhibitors. Furthermore, this work suggests that a number of mammalian proteins may be tyrosine phosphorylated by CK2 (119). Investigation of the effects of CK2 autophosphorylation could lead to increased understanding of CK2 regulation, however the possibility of CK2 acting as a dual specificity kinase in mammalian cells adds greatly to the complexity of its signalling.

1.4 CK2 in the Cell Cycle

CK2 has been implicated in every stage of cell cycle progression, and catalyzes the phosphorylation of several proteins crucial to the successful production of daughter cells. In yeast, genetic studies have shown requirements for CK2 for progression through the G1/S and G2/M transitions (36). In mammalian cells, antisense oligonucleotides against CK2 subunits, microinjection of CK2 antibodies, and inhibitors of CK2 can all inhibit cell cycle progression, suggesting that mammalian cells require CK2 for the G0/G1, G1/S, and G2/M transitions (74, 75, 96). Studies outlining the interactions and effects of CK2 activity on cell cycle proteins have further confirmed the importance of

CK2 throughout the cell cycle. Cell cycle progression is largely mediated by the controlled activation and deactivation of Cyclin-dependent kinases (Cdks), including Cdk4/Cyclin D and Cdk2/Cyclin E at the G1/S transition, and Cdk1/Cyclin B at the G2/M transition (89). These kinases are activated at the appropriate times in part by the activity of the Cdk-activating kinase (CAK), an enzyme consisting of Cdk7, Cyclin H, and MAT1 (ménage à trois 1) (73). Even at this earliest stage of cell cycle progression signalling, CK2 has a role. The CK2α subunit forms a complex with Cyclin H (30), and phosphorylates the cyclin at serine 315 (106). This phosphorylation event has no effect on CAK complex formation, but is critical for full CAK activity (106).

1.4.1 G1/S

The signal to begin DNA replication and prepare for cell division is transduced from extracellular growth signals through the G1/S CDKs, leading to hyperphosphorylation of pRB and transcription of vital cell cycle genes due to release of E2F transcription factor inhibition (108). Once the initial stimulus for growth has been transduced to the G1/S Cdks, CK2 has additional roles in regulating the initiation of DNA replication and preparation for division (Figure 1.3). The most well-studied role for CK2 in G1/S is its regulation of the tumour suppressor protein p53, a transcription factor involved in DNA damage signalling that can elicit both cell cycle arrest and induction of apoptosis (108). p53 is phosphorylated by CK2 at serine 392 in response to UV radiation-induced DNA damage, resulting in increased DNA binding and transcriptional activation (58, 59). In response to DNA damage caused by UV light, CK2 forms an interaction with the FACT complex (consisting of SSRP1 and hSPT16), resulting in increased CK2 activity towards

p53 (59, 60). SSRP1 is also a substrate of CK2, and phosphorylation decreases SSRP1 binding to DNA, halting transcription in the event of DNA damage (67). In undamaged cells, p53 is constantly produced and degraded, and the degradation of p53 is induced by MDM-2 (murine double minute clone 2), which targets p53 for processing by the 26S proteasome. In the presence of cellular stress, the interaction between MDM-2 and p53 is disrupted, leading to stabilization of p53, which can then elicit a cellular response (108). MDM-2 is also a substrate of CK2, which phosphorylates it at serines 269 (3, 37), 267 (50), and 260 (3). CK2 phosphorylation at serine 267 leads to reduced ability to direct p53 degradation (50). Phosphorylation at serine 269 has been shown to decrease MDM-2 binding to pRB, and expression of phosphorylation-site mutants of MDM-2 cause altered growth (38). Treatment of cells with the CK2 inhibitor TBB (4,5,6,7-tetrabromobenzotriazole) leads to induction of p53 and its transcriptional target genes (3), providing additional evidence of the importance of CK2 regulation of p53 signalling.

Another mechanism by which CK2 regulates G1/S signalling is through interaction and phosphorylation of Cdk inhibitory proteins. $p21^{WAF1/CIP1}$, an potent inhibitor of Cdk2 whose expression is induced by p53 activation (108), binds to CK2β (39). CK2 can phosphorylate $p21^{WAF1/CIP1}$ in a CK2β-dependent manner, indicating that CK2β acts to target the catalytic subunits to this substrate (101). $p27^{KIP1}$, another Cdk2 inhibitor, is also phosphorylated by CK2 in a CK2β-dependent manner (113). The effects of CK2 phosphorylation of these substrates has not been determined, but suggests that CK2 can regulate progression through G1/S though multiple mechanisms.

Figure 1.3

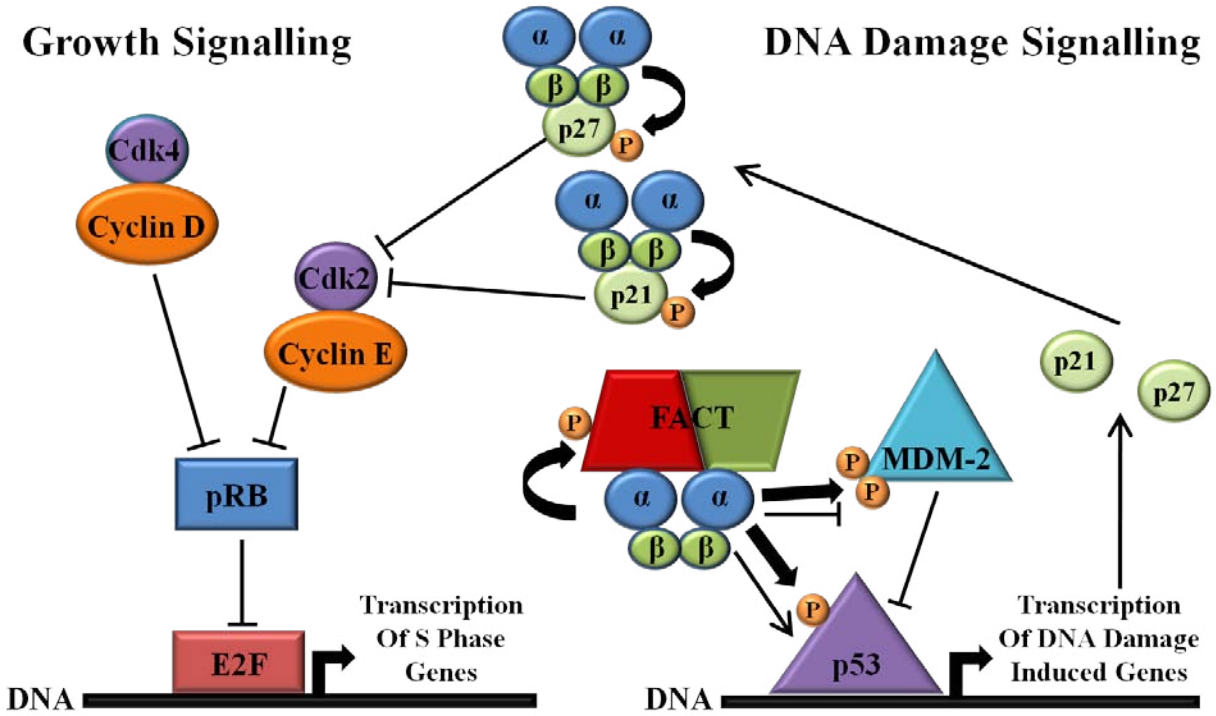
Growth Signalling
DNA Damage Signalling
α
α
β
β
p27
P
Cdk4
Cyclin D
Cdk2
Cyclin E
p21
pRB
FACT
MDM-2
p21
p27
E2F
Transcription
Of S Phase
Genes
DNA
p53
Transcription
Of DNA Damage
Induced Genes
DNA

Figure 1.3 CK2 signaling at the G1/S transition. Positive growth signals lead to activation of the S phase Cdks, Cdk2 and Cdk4, which inhibit pRB binding to E2F transcription factors. Once pRB dissociates from E2F proteins, they induce the transcription of genes required for DNA replication. However, in the presence of DNA damage, the cell will arrest before replication through the action of p53. When DNA damage is sensed, CK2 binds to the FACT complex and phosphorylates SSRP1, leading to decreased transcription in the presence of DNA damage. Binding to FACT targets CK2 kinase activity towards p53. Phosphorylation of p53 by CK2 increases DNA binding and transcription of DNA-damage induced genes. p53 levels are regulated by degradation through binding to MDM-2, which targets p53 to the 26S Proteasome. After DNA damage is detected, CK2 phosphorylation of MDM-2 leads to decreased degradation of p53. p53 induces expression of the Cdk2 inhibitory proteins, p21 and p27, which then bind to and are phosphorylated by CK2. Inhibition of Cdk2 by p21 and p27 decreases transcription of S phase genes in the presence of DNA damage. Thick arrows denote CK2 phosphorylation. Thin arrows denote regulatory effects within the pathway, with pointed arrowheads representing activating effects, and flat arrowheads representing inhibitory effects.

1.4.2 G2/M

Upon completion of DNA replication, the cell then progresses through the G2 stage of the cell cycle and enters mitosis. Entry into mitosis is dependent on the activation of Cdk1, which occurs at the G2/M transition by a combination of phosphorylation, dephosphorylation, and expression of its regulatory cyclin, Cyclin B (68). Upon activation of Cdk1, a massive wave of phosphorylation triggers the onset of mitosis (93). However, if DNA damage is present, Cdk1 activation is delayed due to activation of the DNA damage checkpoint, and the DNA is repaired (48). Mitosis is composed of several stages: in prophase, the DNA condenses to form discrete chromosomes (9), the nuclear envelope breaks down (44), and the centrosomes, also replicated during S phase, migrate to opposite poles of the cell (22). Formation of the mitotic spindle by the centrosomes leads to attachment of spindle fibres to the centromeres of the condensed chromosomes, through a multiprotein complex called the kinetochore (87). The kinetochore is the site of the mitotic checkpoint complex (MCC), the protein effectors of the spindle assembly checkpoint (SAC). The SAC halts mitotic progression until each pair of sister chromatids is properly attached to one kinetochore from each pole (termed amphitelic attachment), ensuring that the daughter cells receive equal amounts of DNA (87). Proper attachment to the kinetochores causes progression into metaphase, at which point the chromosomes line up along the metaphase plate, directly in between the two centrosomes. Once the chromosomes line up, the cell progresses into anaphase, in which protein linkers between sister chromatids are severed, the DNA is decatenated, and the chromosomes divide and are pulled to the centrosomes by the mitotic spindle (111). In telophase, new nuclear

envelopes form around the divided sets of DNA (44), the DNA decondenses (9), and the remainder of the cell divides in two through a process called cytokinesis (111). Through the action of an actin-myosin contractile ring at the midpoint of the cell, the organelles, cytoplasm and plasma membrane are divided equally, producing two identical daughter cells (7).

Several lines of evidence point to specific roles for CK2 in the G2/M transition and mitosis (Figure 1.4). CK2 colocalizes with the mitotic spindle and centrosomes (62, 127), and many proteins involved in mitosis are interacting partners and/or substrates of CK2, including β-Tubulin (31), Cdc25B (116),Tau (6), Condensin (112), PP2A (49), and Microtubule associated proteins (MAP) 1A and 1B (6). Through sequential phosphorylation of Wee1 with Plk1 and Cdk1, CK2 phosphorylation leads to the degradation of Wee1 and onset of mitosis (121). Attenuation of CK2 activity or knockdown of CK2 subunits has been shown to abrogate the SAC after nocodazole treatment, in concert with the p38 MAPK (104). As mentioned above, CK2α and CK2β are both phosphorylated in mitosis (17, 70, 72), suggesting that these subunits in particular are differentially regulated to perform unique roles in mitosis.

Many cell cycle regulatory proteins are phosphorylated by CK2, including Topoisomerase II (24, 28), Cdc34 (11), and Six1 (33) (Table 1.1), however the precise details of these phosphorylation events remain largely unknown. CK2 phosphorylation of Topoisomerase II was initially detected in mammalian cells using the mitosis-specific MPM-2 (mitosis protein monoclonal-2) and 3F3/2 phosphospecific antibodies, which recognize the CK2 sites at serine 1469 and threonine 1342, respectively (24, 28).

Figure 1.4

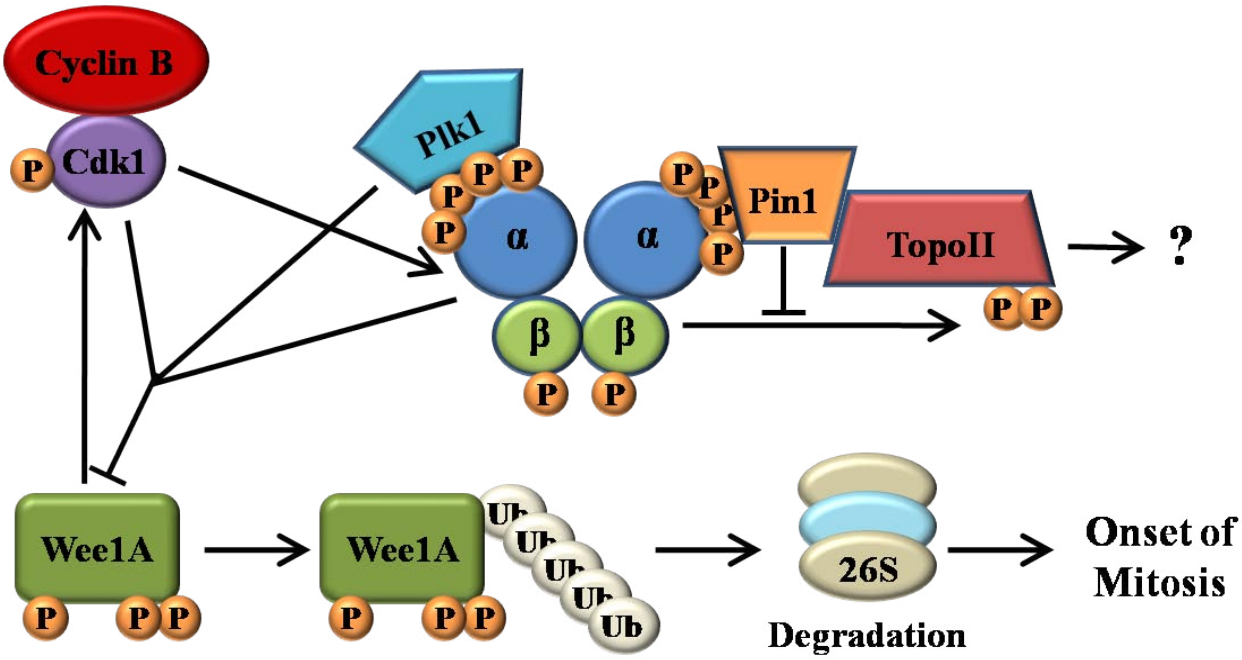

Figure 1.4 CK2 signalling in mitosis. At the onset of mitosis, the master regulatory kinase Cdk1 is activated in part by phosphorylation by Wee1A. Once active, Cdk1 phosphorylates CK2 on both CK2α and CK2β. Phosphorylation of CK2α forms binding sites for both the mitotic kinase Plk1 and the peptidyl-prolyl isomerase Pin1. CK2, Cdk1, and Plk1 participate in hierarchal phosphorylation of Wee1A, leading to its ubiquitination and degradation by the 26S Proteasome. This results in full activation of Cdk1 and the onset of mitosis. Phosphorylation-dependent binding to Pin1 results in decreased phosphorylation of Topoisomerase IIα by CK2 at mitosis-specific sites. The function of this is unknown. Additionally, the role of CK2β phosphorylation has not been determined.

Intriguingly, CK2 can regenerate a number of other MPM-2 reactive phosphoepitopes lost following phosphatase treatment, indicating that there are additional mitotic substrates of CK2, and that the MPM-2 antibody may be instrumental in detecting them (28).

In addition to mitosis-specific substrates, CK2 also forms protein-protein interactions in a mitosis-specific manner (Table 1.2). One of the most intriguing CK2 interactors in mitosis is Pin1, a peptidyl-prolyl isomerase which catalyzes the cis-trans isomerization of proline residues adjacent to phosphorylated serine or threonine (124). Due to the similarities between its isomerization consensus sequence and the phosphorylation consensus sequence of Cdk1, Pin1 binds to several important mitotic phosphoproteins, and may have a role in regulation of mitosis (Reviewed in (78)). Studies in our laboratory have shown that Pin1 binds specifically to CK2α on its phosphorylated C-terminal tail. Furthermore, this interaction forms a complex with Topoisomerase IIα, and leads to decreased CK2-catalyzed phosphorylation of Topoisomerase IIα (85). It is currently unknown whether Pin1 simply binds to the CK2α mitotic phosphorylation sites or actually catalyzes the adjacent prolines, but what is clear is that protein-protein interactions between Pin1 and phosphorylated CK2α can regulate CK2 activity. Recently, a proteomic screen of Plk1 Polo-Box Domain (PBD) interactors in mitosis identified CK2α as a cell cycle-specific, phosphorylation-specific interactor of Plk1 (76). Intriguingly, threonine 344, one of the four mitotic phosphorylation sites located on the CK2α C terminus, fits the consensus sequence for PBD binding (27). Plk1 is an important

TABLE 1.1. Known mitotic substrates of CK2.

Protein	Site	Function	Reference
Topoisomerase IIα	T1342	G2/M DNA Damage Checkpoint, DNA decatenation	(24)
Topoisomerase IIα	S1469	G2/M DNA Damage Checkpoint, DNA decatenation	(28)
Wee1	S121	Serine/threonine kinase	(121)
CDC25B	S186, S187	Dual-specificity phosphatase	(116)
CDC25C	T236	Dual-specificity phosphatase	(107)
PTP-S2	ND	Tyrosine phosphatase	(88)
CDC34	S205, S222, S231, T233, S236	Ubiquitin ligase	(11)
Bdp1	S390, S341, T437, S446	Transcription factor	(53)
Six1	ND	Transcription factor	(33)

ND: Not determined

TABLE 1.2. Known mitotic interactors of CK2.

Protein	Function	Experimental Evidence	Reference
Pin1	Peptidyl-prolyl isomerase	GST Pulldown	(85)
Plk1	Serine/threonine kinase	GST Pulldown	(76)
β-Tubulin	Structural spindle protein	Coimmunoprecipitation	(31)
Wee1*	Serine/threonine kinase	Coimmunoprecipitation	(92, 126)
Chk1*	Serine/threonine kinase	Coimmunoprecipitation	(41)

*Interaction mediated through CK2β

kinase regulating multiple facets of mitotic progression (Reviewed in (118)), and determining the role of this interaction in mitotic progression could shed new light on the role of CK2 in mitosis.

The onset of proteomic studies of mitotic phosphorylation and interaction events have provided additional information regarding the role of CK2 in mitosis. Proteomic investigations have confirmed previous localization studies by identifying CK2 as a component of both the centrosomes (5) and spindle midbody (110). Phosphoproteomic screens have identified additional potential mitotic substrates of CK2, including Septin-2, INCENP, and MAP7 (91). The limitation of proteomics approaches to identify phosphorylation-specific targets and interactors of CK2α is that the phosphorylation sites are not readily available for mass spectrometric analysis. This is due to a lack of lysine residues in the CK2α C-terminus, resulting in a protein fragment too large to ionize efficiently after typical trypsin digestion. For this reason, large mitotic proteomic screens (which typically use only trypsin) have been of limited value to the study of CK2α phosphorylation.

1.5 Objectives

As discussed above, CK2 is instrumental for several cellular processes, including multiple stages in the process of cell proliferation. However, how CK2 is regulated to ensure proper function at various points in the cell cycle is not well understood. In this thesis, we examine the regulation of CK2 during cell division (Figure 1.5). We hypothesize that CK2 is regulated during mitosis, enabling the constitutively active kinase to perform functions unique to cell division.

First, we generated phosphospecific antibodies against the four mitotic phosphorylation sites in the C-terminus of CK2α. As CK2α phosphorylation had previously only been shown in cells arrested in mitosis using the microtubule poison nocodazole (17, 72), these antibodies were used to investigate CK2α phosphorylation during the normal progression of mitosis. We also generated stable cell lines expressing CK2α phosphorylation-site mutant proteins, with the objective of studying the effects of disruption of CK2α phosphorylation on mitotic progression. The results of these studies are discussed in detail in Chapter 2.

Secondly, we examined the subcellular localization of CK2 during mitosis. While previous studies have demonstrated CK2 localization to the mitotic spindle, the subunit specificity of this localization produced mixed results (62, 127). Additionally, the effect of the four mitotic phosphorylation sites in CK2α had not been examined. Using the aforementioned phosphospecific antibodies, we employed immunostaining and confocal microscopy to examine the mitotic localization of unphosphorylated and phosphorylated CK2α. The results of this study are discussed in detail in Chapter 3.

Finally, we examined the role of hierarchal phosphorylation between CK2 and the mitotic master kinase, Cdk1. As mentioned above, as CK2 is an acidophilic kinase, it has the ability to participate in hierarchal phosphorylation events by using a phosphoserine residue as its acidic determinant for phosphorylation (2, 121). In mitosis, this phenomenon results in proper degradation of Wee1A, an important regulator of mitotic entry (121). Intriguingly, these hierarchical phosphorylation events essentially put CK2, a constitutively active kinase, under the strict regulation of Cdk1, and this may in part

explain how CK2 activity towards mitotic substrates is regulated. We were therefore interested in discovering other potential mitotic substrates of CK2 that may be regulated in the same way. Since hierarchal phosphorylation has never been systematically investigated, it was first necessary to determine an optimal consensus sequence for hierarchical phosphorylation involving CK2 and Cdk1, before conducting a genome-wide bioinformatics screen to identify potential mitotic substrates. Results from this work are discussed in detail in Chapter 4.

Overall, these studies have used a variety of approaches to extend our understanding of the role and regulation of CK2 during mitosis. Collectively, the results in this thesis demonstrate that CK2, although largely regarded as constitutively active, is regulated through multiple subtle means, including phosphorylation, localization, and concerted action with differentially regulated kinases. Through these regulatory mechanisms, CK2 is effectively targeted towards mitotic substrates, leading to proper mitotic division and maintenance of genomic stability.

Figure 1.5

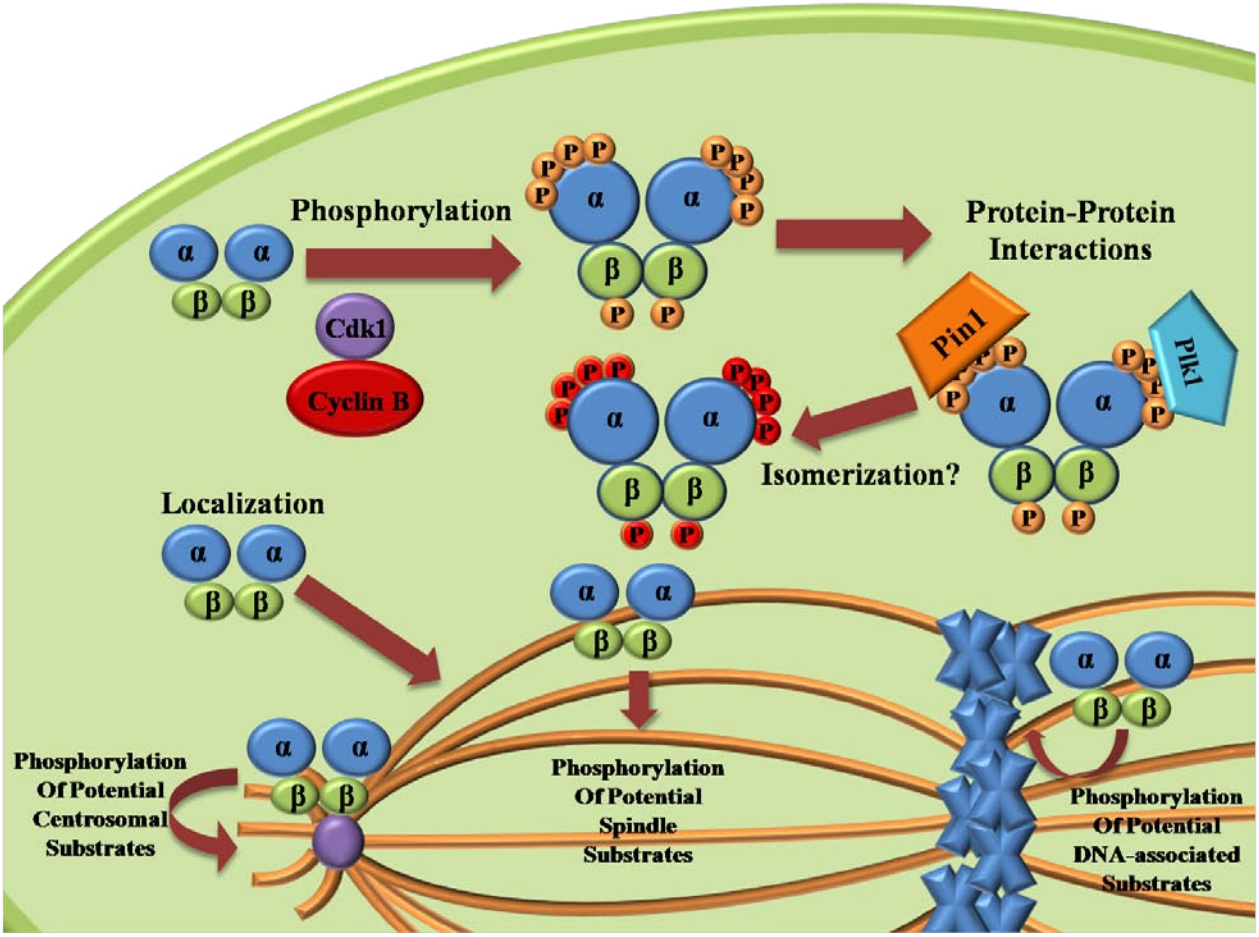
Phosphorylation
Cdk1
Cyclin B
Protein-Protein
Interactions
Pin1
Plk1
Isomerization?
Localization
Phosphorylation
Of Potential
Centrosomal
Substrates
Phosphorylation
Of Potential
Spindle
Substrates
Phosphorylation
Of Potential
DNA-associated
Substrates

Figure 1.5 Schematic of potential mechanisms for the regulation of CK2 during cell division. While CK2 is intrinsically constitutively active, it may be regulated by multiple mechanisms during mitosis. Changes in subcellular localization can lead to association with the centrosomes (in purple) and the mitotic spindle (in orange), where CK2 has a number of interacting proteins and potential substrates. From the mitotic spindle, CK2 could also access several DNA-associated substrates. Condensed mitotic chromosomes are shown in blue. CK2α and CK2β are phosphorylated by Cdk1/Cyclin B at the onset of mitosis. Phosphorylation of CK2α leads to protein-protein interactions with Plk1 and Pin1. It is unknown whether interaction with Pin1 leads to isomerization of prolyl residues adjacent to the CK2α phosphorylation sites. As well, the interplay between these different modes of regulation has not been studied.

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Chapter 2: Evidence for Regulation of Mitotic Progression through Temporal Phosphorylation and Dephosphorylation of CK2α[1]

2.1 Introduction - Proper progression through mitosis is mediated by a complex web of signalling pathways that ensure faithful division of genetic material, and deregulation of these pathways can lead to aneuploidy and genetic instability (15). Protein kinase CK2 is a pleiotropic serine/threonine kinase that is upregulated in a variety of human cancers (Reviewed in (12)) and possesses oncogenic properties in mice and fibroblast cultures (18, 29). The kinase is generally found as a tetramer, with two catalytic subunits (CK2α and/or CK2α') and two regulatory subunits (CK2β) (11). CK2 is involved in signalling pathways controlling multiple cellular processes, including cell cycle control and cell survival (Reviewed in (19)). In these pathways, CK2 has a multitude of different interacting proteins and substrates, and subsequently, information on the precise regulation of CK2 has been elusive.

Expression of CK2 is essential for viability in both yeast and slime mold (16, 30), and is required for progression through the G1/S and G2/M transitions of the yeast cell cycle (13, 30). In mammalian cells, there are requirements for CK2 at the G0/G1, G1/S, and G2/M phases of the cell cycle (21, 22, 31). CK2α, one of the catalytic subunits of CK2, contains four proline-directed phosphorylation sites (T344, T360, S362, and S370) that are phosphorylated in nocodazole-arrested cells (4, 20). The reactions are catalyzed *in vitro* by the mitotic cyclin-dependent kinase Cdk1, which is believed to be the kinase responsible in cells (4). These phosphorylation sites are located on the extended C-terminal tail of CK2α and are therefore not present in CK2α' (25). This difference

[1] A modified version of this chapter has been published: St-Denis NA, Derksen DR, Litchfield DW. **Evidence for Regulation of Mitotic Progression through Temporal Phosphorylation and Dephosphorylation of CK2α** (2009). Molecular and Cellular Biology 29(8):2068-2081.

between isoforms suggests some functional specialization for the catalytic subunits of CK2. Interestingly, while mice lacking CK2α' are viable (40), CK2α knockout results in embryonic lethality (23). The CK2α C-terminal phosphorylation sites are conserved in birds and mammals, further supporting the idea that they play an important role in regulating the function of CK2 (25).

To examine the phosphorylation of CK2α in mitosis, we generated phosphospecific antibodies against its phosphorylation sites. We show that CK2α is phosphorylated in mitotic cells. This phosphorylation occurs mainly in prophase and metaphase, decreases through anaphase, and is absent in telophase and cytokinesis. To gain insight on the function of CK2α phosphorylation in mitosis, cell lines with tetracycline-regulated expression of phosphorylation-site mutants of CK2α, either with phosphomimetic aspartic acid or glutamic acid substitutions (CK2α-4D, CK2α-4E) or with non-phosphorylatable alanine substitutions (CK2α-4A) were examined. Expression of CK2α phosphomimetic mutant proteins resulted in aberrant centrosome amplification, chromosomal segregation defects, and loss of mitotic cells through mitotic catastrophe. Non-phosphorylatable CK2α expression did not result in these effects, but cells showed decreased ability to arrest following spindle insult by nocodazole treatment. Taken together, these results show that proper temporal regulation of CK2α phosphorylation is required for proper mitotic progression, and highlight a role for CK2α phosphorylation in maintenance of spindle integrity and control of cell division.

2.2 Materials and Methods

Antibodies - Polyclonal antibodies against phosphorylated CK2α were raised in New Zealand White rabbits against phosphorylated peptides (pT344: CANSSVPpTSGG; pT360/pS362: CISSVPpTPpSPL; pS370: CRRRLAGpSPVI) coupled to Keyhole Limpet Hemocyanin by Covance Research Products, Inc. (Denver, PA). Non-phosphospecific antibodies were immunodepleted from the antisera on SulfoLink Resin (Pierce) conjugated to non-phosphorylated versions of the above peptides. Phosphospecific antibodies were isolated from the resultant flow through by affinity purification with phosphorylated peptides. Polyclonal anti-CK2α, anti-CK2β and anti-Cdk1 antiserum have been previously described (22). The monoclonal antibody 12CA5 which reacts against the HA epitope was purchased from Roche. The culture supernatant from the hybridoma producing the 9E10 monoclonal antibodies directed against the Myc epitope, developed by J.M. Bishop was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Goat CK2α, Phospho-Histone H3 (Serine 10), Cyclin B1 and Cytochrome C antibodies were purchased from Santa Cruz Biotechnology. Pericentrin antibodies were purchased from AbCam. Monoclonal antibodies against β-tubulin were a generous gift from Dr. Lina Dagnino (Department of Pharmacology, University of Western Ontario). Goat anti-rabbit (GAR) or goat anti-mouse (GAM) horseradish peroxidase (HRP) secondary antibodies were purchased from BioRad. FITC-GAR was from Sigma, and Texas Red-GAR and AlexaFluor® 488-GAM were from Molecular Probes. Fluorescent secondary antibodies for immunoblot detection were purchased from LI-COR Biosciences.

Plasmid Constructs - The CK2α-HA/Myc-CK2β bidirectional plasmid in pBI (Clontech) and CK2α phosphorylation site mutant proteins in pRc/CMV (Invitrogen) have been previously described (31, 42). To introduce the CK2α phosphorylation site mutations into the tetracycline-responsive, bidirectional pBI vector, the pRc/CMV vectors were cut with BstBI and BlpI restriction endonucleases (New England Biolabs) to release a 1040 base pair insert containing the mutations at the C-terminal phosphorylation sites. The CK2α-HA/Myc-CK2β bidirectional plasmid was digested in the same manner, and the mutated inserts were ligated into the wild type plasmid, thus replacing a wild type CK2α C-terminus with a mutant C-terminus. All plasmids were verified by DNA sequencing.

Generation and Maintenance of Cell Lines - UTA6 cells were derived from the human osteosarcoma U2OS cell line, and express the tetracycline trans-activator (tTA) fusion protein (a generous gift from Dr. Christoph Englert, Forschungszentrum Karlsruhe, Germany) (9). Cell lines expressing CK2α-HA and CK2α-KD-HA (Kinase Dead) have been previously described (42). Cell lines with tetracycline-regulated expression of HA-tagged CK2α phosphorylation site mutants were generated by co-transfection of CK2α-4D-HA, CK2α-4E-HA, or CK2α-4A-HA with the pTK-hyg plasmid (Clontech) in the presence of tetracycline. Drug selection with 500 μg/mL hygromycin and 460 μg/mL G418 (Life Technologies Inc.) began 48 hours after transfection. Once stably transfected colonies had formed, they were picked and transferred to 96-well dishes. Colonies were expanded and tested for tight inducible expression of CK2α phosphorylation site mutants by western blot analysis. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) with 10% fetal calf

serum (Invitrogen), antibiotic supplements (0.1 mg/mL streptomycin and 100 units/ml penicillin) (Life Technologies Inc.), and 1.5 mg/mL tetracycline (Sigma). To achieve cell synchronization in S phase, cells were treated by double thymidine block with two 16 hour treatments with 2mM thymidine (Sigma) separated by a 10 hour incubation without thymidine. To achieve cell synchronization in G2/M, cells were released from S phase arrest for 8 hours and then treated with 40 ng/mL nocodazole (Sigma) for 18 hours.

Cell Lysis and Immunoprecipitation - Cells were lysed on ice in NP-40 lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 1 µg/mL Pepstatin A, 1 µg/mL Leupeptin, 1mM PMSF, 1mM NaF, 4mM Na_2VO_4). The lysates were sonicated on ice in three 10s bursts and then centrifuged in a Beckman TL100.2 rotor at 55000 rpm for 15 min. For immunoprecipitation, 1 mg of total cell lysate was incubated with 2 µL of 12CA5 anti-HA antibody or 5 µL of anti-Cdk1 antisera bound to Protein A Sepharose for one hour at 4˚C. Beads were washed four times in lysis buffer and proteins were eluted in boiling sample buffer. Where dephosphorylation was required, lysates were incubated with 1 µL of Lambda Phosphatase (New England Biolabs) for 30 minutes at 37°C.

Immunoblot analysis - The protein concentration of each sample was determined using the BCA protein assay (Pierce). Equal amounts of protein lysate were separated by SDS-PAGE using the method of Laemmli (19). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim) for 1 hour at 15 V and 0.3 A using the Trans-blot Semi-Dry Electrophoretic transfer apparatus (Bio-Rad). Immunoblotting was performed by blocking one hour in 5% BSA in TBST, followed by overnight incubation with the primary antibody. Immune complexes were detected either by incubation with HRP-linked secondary antibodies and detection by

chemiluminescence, or incubation with fluorophore-linked secondary antibodies and detection on a LI-COR Near-Infrared Fluorescent Scanner and Odyssey V3.0 software.

Kinase Assays - CK2 activity was measured in whole cell extracts using a synthetic peptide substrate (RRRDDDSDDD) (23). Assays were performed for 5 minutes or 10 minutes at 30°C in a final reaction volume of 30 µl containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 0.1 mM ATP (specific activity 500-1000 cpm/pmol, ICN) and 0.1 mM substrate peptide. Reactions were initiated by the addition of 2 µg or 4 µg of cell extract. The reactions were terminated by spotting 10 µL of the reaction mixture on P81 phosphocellulose paper. Samples were washed 4 times in 1% phosphoric acid and once in 95% ethanol. Activity was detected with a Beckman LS 5801 scintillation counter. Cdk1 activity was measured using Histone H1 (Calbiochem) as described in (22).

Growth Curve Construction - CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA expressing cells were seeded in 6-well dishes at a starting density of 20 000 cells per well. Protein expression was induced and complete media was added in the presence or absence of 1.5 µg/ml tetracycline. The media was changed every 3 days. On days 0, 1, 3, 5 and 7, the cells were harvested with 5 mM EDTA in PBS and counted with a haemocytometer. Trypan blue (Gibco) was used to distinguish viable from non-viable cells.

Cell Cycle Analysis - CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA expressing cells were arrested in S phase by double thymidine block. Upon release from thymidine block, protein expression was induced and complete media was added to

the cells in the presence or absence of 1.5 µg/ml tetracycline. For 24 hours, floating and adherent cells were collected every two hours with 5mM EDTA in PBS and fixed in 70% ethanol overnight at 4 º C. Cells were then washed with PBS and stained with propidium iodide (PI) staining solution (0.1% sodium citrate, 0.1% Triton X-100, 50 µg/mL PI (Sigma), and 0.1 mg/mL DNAse-free RNAse A (Sigma)) for 20 minutes at 37˚C. Nocodazole arrested cells were fixed and stained as above. Cells stained with Phospho-Histone H3 were harvested 12 hours after thymidine release and induction then fixed as above. After washes with PBS, PBS + 0.5% Triton X-100, and PBS + 5% BSA (PBS-BSA), cells were incubated two hours with 1 µg/mL Phospho-Histone H3 in PBS-BSA. Positive cells were detected using goat anti-rabbit FITC (1:200) (Sigma) in PBS-BSA. Cells were then counterstained with PI as above. Cells were analyzed on a Becton Dickinson fluorescence-activated cell sorter (FACScan) using Cell Quest Pro software (Becton Dickinson). In each sample, 20,000 events in a specific gated region were counted. Data analysis was carried out using FlowJo software (Tree Star).

Immunostaining and Microscopy - Cells used for immunostaining were grown on Poly-L-Lysine coated coverslips (Bio-Rad). For mitochondrial staining, cells were incubated for 30 minutes with 200 nM MitoTracker® Deep Red 633 FM prior to fixation. Following fixation with 50:50 Methanol:Acetone, coverslips were incubated in 0.1% Glycine in PBS and blocked in 5% FBS in PBS. Primary and secondary antibody incubations were performed at 37˚C for 1 hour and 30 minutes respectively. After washing with PBS, DNA was stained with DAPI and coverslips were mounted onto microscope slides with AirVol. Cells were visualized on a Zeiss META 510 LSM

Confocal microscope. Z-series images of 0.6 µm thickness were captured and processed with Zeiss software and Adobe Photoshop.

Trypan Blue Viability Assays - Cells were arrested by thymidine block and protein expression was induced as above. Twenty-four hours after induction, floating and adherent cells were collected using 5mM EDTA in PBS and stained with 0.4% Trypan Blue Stain (Gibco). Total and non-viable cells were counted with a haemocytometer.

2.3 Results

Generation and characterization of CK2α phosphospecific antibodies

To investigate mitotic phosphorylation of CK2α, we generated polyclonal antibodies targeting the four C-terminal phosphorylation sites. Three antibodies were made, targeting the T344, T360/S362, and S370 phosphorylation sites respectively. Following affinity purification, the antibodies were tested for phosphospecificity. Phosphorylation of CK2α can be detected after gel electrophoresis by its slight mobility decrease (Figure 2.1A, CK2α immunoblot). Lysates from cells arrested in S phase by thymidine block show no mobility shift due to phosphorylation, and accordingly, show no reactivity when immunoblotted with CK2α phosphospecific antibodies (Figure 2.1A, Thymidine lanes). Lysates from cells arrested in mitosis by nocodazole treatment show robust reactivity towards CK2α phosphospecific antibodies at a position corresponding to the phosphorylated portion of the total CK2α (Figure 2.1A, Nocodazole lanes). Additionally, all three phosphospecific antibodies show no cross-reactivity to unphosphorylated CK2α. To confirm that these antibodies are specifically detecting phosphorylation, we next immunoblotted lysates from nocodazole arrested cells after dephosphorylation with λ-Phosphatase. As shown in Figure 2.1B, treatment with λ-Phosphatase resulted in loss of the mobility-shift associated with CK2α phosphorylation and loss of reactivity to CK2α phosphospecific antibodies. To test the ability of the phosphospecific antibodies to detect phosphorylated CK2α in cells, nocodazole-arrested cells were fixed and immunostained using CK2α phosphospecific antibodies. DNA was stained with DAPI. All three antibodies exclusively stain mitotic cells, recognized by the condensed nature of the chromosomes (Figure 2.1C). The signal is specific to

Figure 2.1

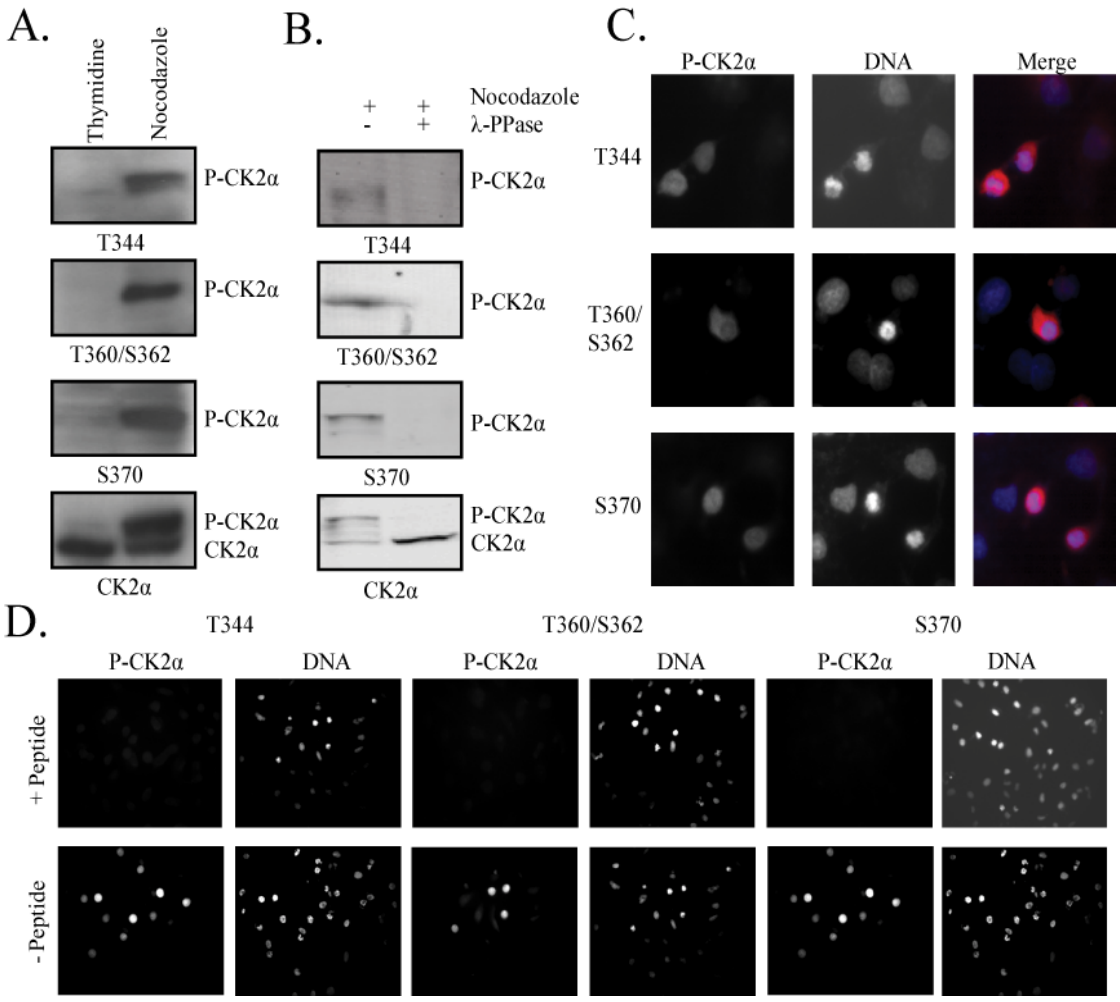
A.
Thymidine
Nocodazole
P-CK2α
T344
P-CK2α
T360/S362
P-CK2α
S370
P-CK2α
CK2α
CK2α
B.
+ +
- +
Nocodazole
λ-PPase
P-CK2α
T344
P-CK2α
T360/S362
P-CK2α
S370
P-CK2α
CK2α
CK2α
C.
P-CK2α
DNA
Merge
T344
T360/
S362
S370
D.
T344
T360/S362
S370
P-CK2α
DNA
P-CK2α
DNA
P-CK2α
DNA
+ Peptide
- Peptide

Figure 2.1 Generation of CK2α phosphospecific antibodies. A. Lysates from cells arrested in S phase by double thymidine block or mitosis by nocodazole treatment were immunoblotted with CK2α phosphospecific antibodies targeting phosphorylated T344, T360/S362, or S370. Total CK2α was detected using a CK2α antibody. B. Lysates from nocodazole-arrested cells were left untreated or dephosphorylated using λ-Phosphatase before immunoblotting as in A. C. Nocodazole-arrested cells were fixed and immunostained using CK2α phosphospecific antibodies. DNA was stained with DAPI. Mitotic cells are recognizable by the condensation of chromosomes. Magnification: 40X. D. CK2α phosphospecific antibodies were preincubated with phosphorylated peptides corresponding to the appropriate phosphorylation site before immunostaining of nocodazole-arrested cells as in C. DNA is stained with DAPI. Magnification: 20X.

phosphorylated CK2α, as preincubation of the antibodies with the phosphorylated peptides used in antibody generation completely blocks detection by immunostaining (Figure 2.1D). To test for cross-reactivity between the three antibodies, we also preincubated each antibody with peptides targeting the other phosphorylation sites. When peptides targeted the same phosphorylation site as the antibody, antibody reactivity was blocked, but peptides targeting phosphorylation sites not targeted by a particular antibody did not inhibit antibody reactivity (Figure 2.2). Taken together, these results confirm that the phosphospecific antibodies are specific to the CK2α C-terminal phosphorylation sites.

CK2α is phosphorylated in prophase and metaphase and dephosphorylated during anaphase

While CK2α has long been known to be maximally phosphorylated in nocodazole-treated cells on the basis of its shift in electrophoretic mobility (4, 20), phosphospecific antibodies provide new opportunities to directly evaluate the extent of phosphorylation as mitosis progresses in a non-arrested cell. To test whether CK2α phosphorylation occurs in all mitotic cells, we compared the proportion of CK2α phosphorylated in cells undergoing normal mitotic progression versus those arrested in mitosis by nocodazole treatment. U2OS cells were arrested in S phase by double thymidine block and released into the cell cycle for 9, 12, or 15 hours. At these time points, mitotic cells were collected by mitotic shake-off and analyzed for CK2α phosphorylation using the phospho-S370 antibody (Figure 2.3A). When equal amounts of cell lysates were subjected to western blot analysis, CK2α was found to be phosphorylated in mitotic cells, albeit at lower levels than in cells treated with nocodazole. This result is also shown using a total CK2α antibody, with mitotic cells exhibiting the characteristic shift seen upon

Figure 2.2

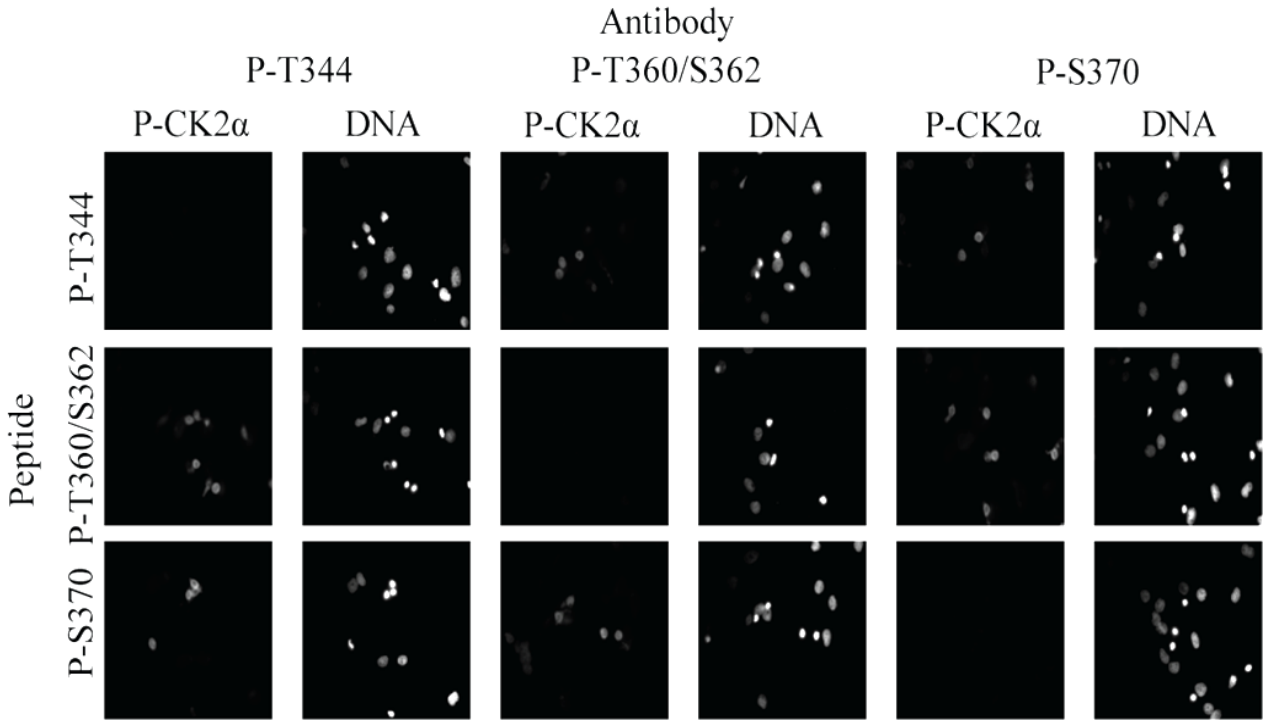

Figure 2.2 CK2α phosphospecific antibodies do not display phosphorylation site cross-reactivity. CK2α phosphospecific antibodies were preincubated with phosphorylated peptides corresponding to all three antibodies generated before immunostaining of nocodazole-arrested cells. DNA is stained with DAPI. Mitotic cells are recognizable by the condensation of chromosomes. Magnification: 40X.

Figure 2.3

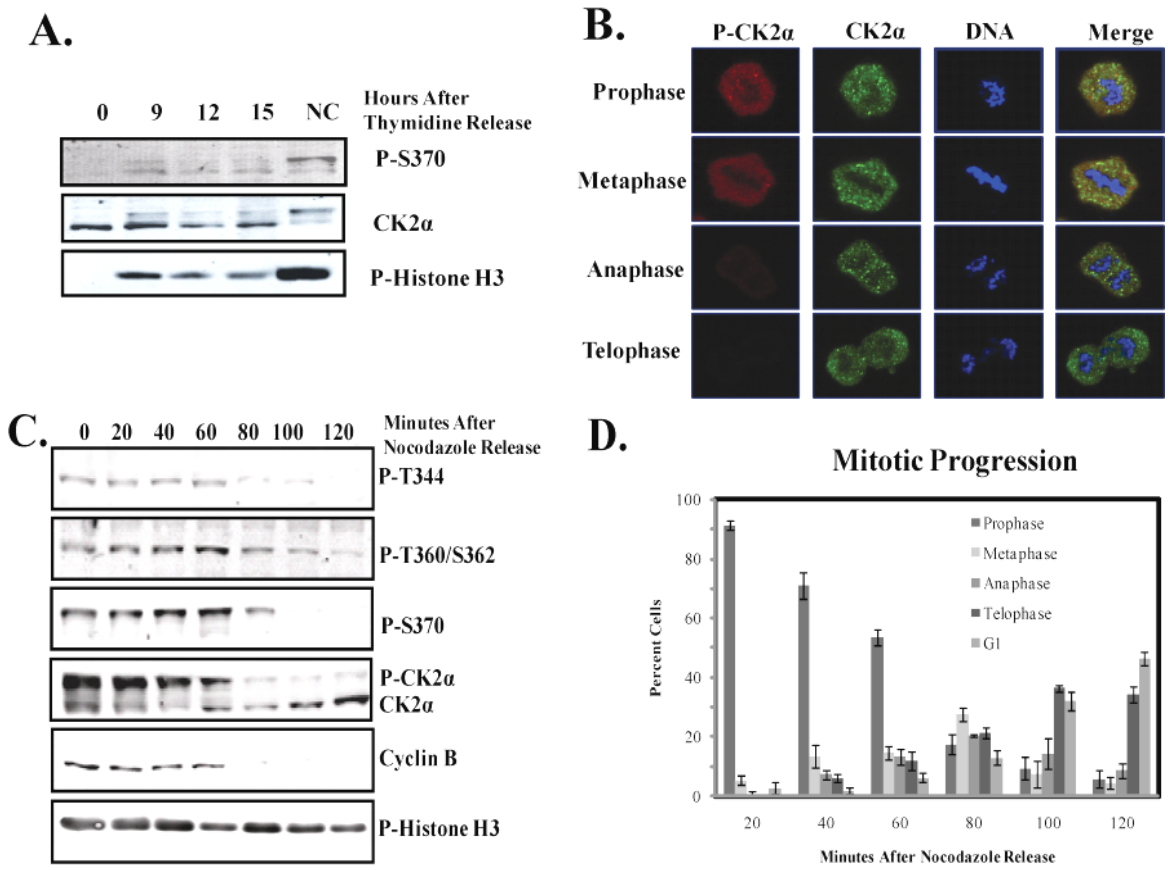
A.
0
9
12
15
NC
Hours After Thymidine Release
P-S370
CK2α
P-Histone H3
B.
P-CK2α
CK2α
DNA
Merge
Prophase
Metaphase
Anaphase
Telophase
C.
0 20 40 60 80 100 120
Minutes After Nocodazole Release
P-T344
P-T360/S362
P-S370
P-CK2α
CK2α
Cyclin B
P-Histone H3
D.
Mitotic Progression
Percent Cells
100
80
60
40
20
0
Prophase
Metaphase
Anaphase
Telophase
G1
20
40
60
80
100
120
Minutes After Nocodazole Release

Figure 2.3 CK2α phosphorylation occurs in mitotic cells during prophase and metaphase. A. U2OS cells were synchronized in S phase by double thymidine block and released into mitosis for 9, 12 or 15 hours before harvest. Lysates were immunoblotted using the P-S370 CK2α phosphospecific antibody. Total CK2α is also shown. Mitotic cells were detected using a Phospho-Histone H3 (Ser 10) antibody. NC, Nocodazole-treated. B. U2OS cells were synchronized as in A, fixed 12 hours after thymidine release, and immunostained with antibodies against phosphorylated CK2α (Red) and total CK2α (Green). DNA was stained with DAPI. Magnification: 63X. C. U2OS cells were arrested in mitosis by nocodazole treatment, washed, and harvested at 20 minute intervals after removal of nocodazole. Lysates were immunoblotted with antibodies against phosphorylated CK2α, total CK2α, Cyclin B1 to detect the onset of anaphase, and Phospho-Histone H3 (Serine 10) to detect mitotic cells. D. Cells were arrested in mitosis as in C, and plated on slides following release. After fixation and DAPI staining, cells were scored for mitotic stage based on DNA morphology. At least 100 cells were scored for each time point in each of three replicate experiments. The error bars indicate one standard deviation from the mean.

phosphorylation. Phospho-Histone H3 (Serine 10) was used as a marker of mitosis.

We next examined whether CK2α phosphorylation was occurring throughout mitosis. To achieve this, U2OS cells were arrested in S phase by double thymidine block, released from the block for 12 hours and then fixed for immunostaining with phosphospecific and total CK2α antibodies. As shown in Figure 2.3B, cells in prophase and metaphase show robust phospho-CK2α staining. The signal decreases in anaphase and is undetectable in telophase and cytokinesis. To confirm this result, cells were arrested in prometaphase by nocodazole treatment to enable tracking of the phosphorylation in a synchronized population. After removal of nocodazole, cells were harvested at 20 minute intervals to track CK2α phosphorylation through mitosis by western blot analysis using CK2α phosphospecific antibodies (Figure 2.3C). Phosphorylation was initially strong, and decreased after 80 minutes of progression. This result is also shown using a CK2α antibody, with mitotic cells exhibiting the characteristic shift seen upon CK2α phosphorylation. This correlated well with the onset of anaphase, as Cyclin B expression decreases just prior to the onset of CK2α dephosphorylation. Histone H3, which remains phosphorylated at serine 10 until the completion of cytokinesis (7), served as a marker for late mitosis. To determine the average mitotic stage of cells at each time point, cells were incubated on coverslips after release from nocodazole arrest and sorted into mitotic stages based on DNA morphology (Figure 2.3D). The majority of cells remained in prophase and metaphase until the 60 minute time point and progressed into anaphase at 80-100 minutes after release. From these results, we conclude that CK2α is phosphorylated in mitotic cells, but at lower levels than in nocodazole-arrested cells. This phosphorylation is temporally regulated,

with maximal phosphorylation in prophase and metaphase and dephosphorylation at the onset of anaphase.

Characterization of cell lines with inducible expression of CK2α phosphorylation site mutants

The above results indicate that temporal regulation of CK2α phosphorylation may be important for mitotic progression. To study the role of the phosphorylation of CK2α in cell cycle progression, all four proline-directed phosphorylation sites unique to the CK2α C-terminus (T344, T360, S362, and S370) were mutated to either aspartic acid (CK2α-4D) or glutamic acid (CK2α-4E), in order to mimic the phosphorylated state. Additionally, the phosphorylation sites were mutated to alanine (CK2α-4A) in order to generate a non-phosphorylatable form of CK2α (Figure 2.4A). UTA6 cells with stable, tetracycline-regulated expression of CK2α phosphorylation site mutants were generated using a bidirectional plasmid expressing both an HA-tagged CK2α phosphorylation site mutant and Myc-tagged CK2β (Figure 2.4B). This bidirectional system has the advantage of maintaining the stoichiometry of the CK2 tetramer, and tetracycline regulation ensures tight control over expression of the mutant proteins. Myc-CK2β coimmunoprecipitates with CK2α-HA, indicating that the exogenously expressed proteins can interact in the cell, and are likely incorporated into mixed CK2 tetramers with endogenous CK2 (Figure 2.4C). In cell lysates, the presence of CK2α phosphorylation site mutants caused an increase in kinase activity comparable to the increase seen with wild type CK2α-HA. Thus, these phosphorylation site mutations do not appear to directly affect the enzymatic activity of CK2α (Figure 2.4D).

Figure 2.4

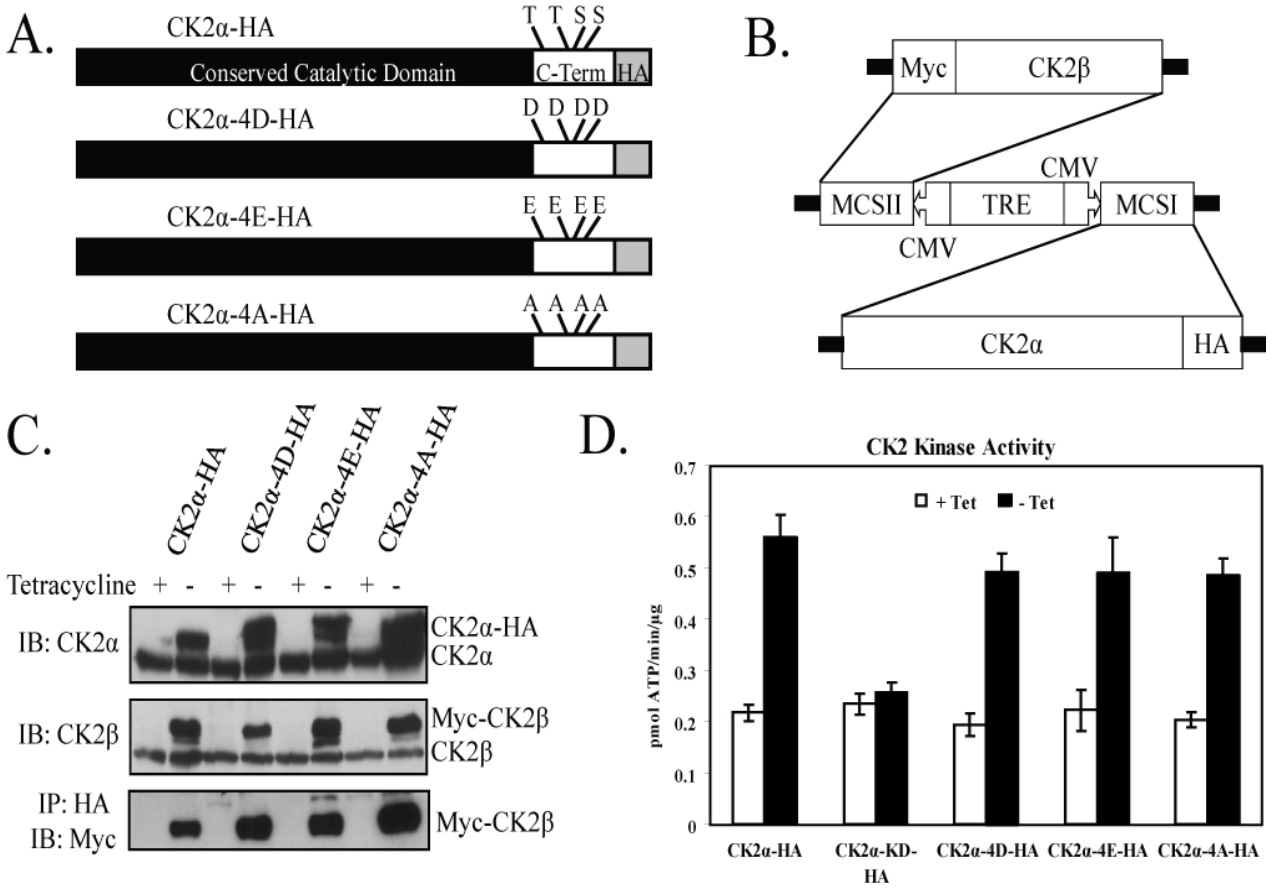
A.
CK2α-HA
T T S S
Conserved Catalytic Domain
C-Term
HA
CK2α-4D-HA
D D DD
CK2α-4E-HA
E E EE
CK2α-4A-HA
A A AA
B.
Myc
CK2β
CMV
MCSII
TRE
MCSI
CMV
CK2α
HA
C.
CK2α-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA
Tetracycline + - + - + - + -
IB: CK2α
CK2α-HA
CK2α
IB: CK2β
Myc-CK2β
CK2β
IP: HA
IB: Myc
Myc-CK2β
D.
CK2 Kinase Activity
+ Tet
- Tet
pmol ATP/min/μg
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CK2α-HA
CK2α-KD-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA

Figure 2.4 Inducible expression of CK2α phosphorylation-site mutants. A. CK2α C-terminal phosphorylation sites (T344, T360, S362 and S370) were mutated to aspartic acid (CK2α-4D-HA), glutamic acid (CK2α-4E-HA), or alanine (CK2α-4A-HA). B. HA-tagged CK2α mutants were stably expressed from a tetracycline-regulated bidirectional vector along with Myc-tagged CK2β. MCSI/II, Multiple Cloning Sites I and II; CMV, Cytomegalovirus promoter; TRE, Tetracycline-responsive Element. C. Inducible expression of tetrameric complexes containing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, CK2α-4A-HA and Myc-CK2β. Cells were incubated for 24 hours in the presence (+) or absence (-) of tetracycline. Equal amounts of lysate were resolved by SDS-PAGE and subjected to immunoblot analysis with antibodies targeted against CK2α and CK2β, or immunoprecipitated with a 12CA5 (anti-HA) antibody and immunoblotted with an anti-Myc antibody. D. Lysates were prepared from cells with tetracycline-regulated expression of CK2α-HA, CK2α-KD-HA (Kinase dead), CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA, cultured in the presence (+ Tet, white bars) or absence (- Tet, black bars) of tetracycline for 24 hours. Lysates were incubated with a synthetic peptide substrate of CK2 (RRRDDDSDDD) and [γ-^{32}P]-ATP. Kinase activities are the average of four determinations. The error bars indicate one standard deviation from the mean.

Expression of CK2α phosphorylation site mutants results in decreased proliferation

To determine if mutation of the four conserved CK2α phosphorylation sites had any effect on cell proliferation, we analyzed the growth of cells expressing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, or CK2α-4A-HA in combination with Myc-CK2β. Protein expression was induced by removal of tetracycline from the culture medium and cells were counted over a seven-day period to examine proliferative capacity compared to cells grown in the absence of CK2α overexpression. Cells expressing wild type CK2α-HA showed a slight decrease in proliferation over the seven day period, consistent with previous results (38). In the three phosphorylation site mutant cell lines, the proliferation defects were more marked compared to their non-expressing counterparts (Figure 2.5A). To ensure that the observed growth defects were not artefacts from random integration events in the construction of the stable cell lines, growth curves were also prepared using additional cell lines expressing each mutant protein. These cell lines also exhibited decreased growth upon expression of CK2α mutant proteins (Figure 2.5B).The decreased proliferation observed upon expression of CK2α phosphorylation site mutant proteins suggests that CK2α phosphorylation does have a functional effect on cellular proliferation.

As expression of CK2α phosphorylation site mutant proteins caused varied decreases in proliferation, we next sought to determine if any specific point in the cell cycle was perturbed upon expression. Cells expressing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA were grown in the presence or absence of tetracycline and arrested in S phase by double thymidine block. Upon release from a double thymidine block, CK2α mutant protein expression was induced (Figure 2.6A).

Figure 2.5

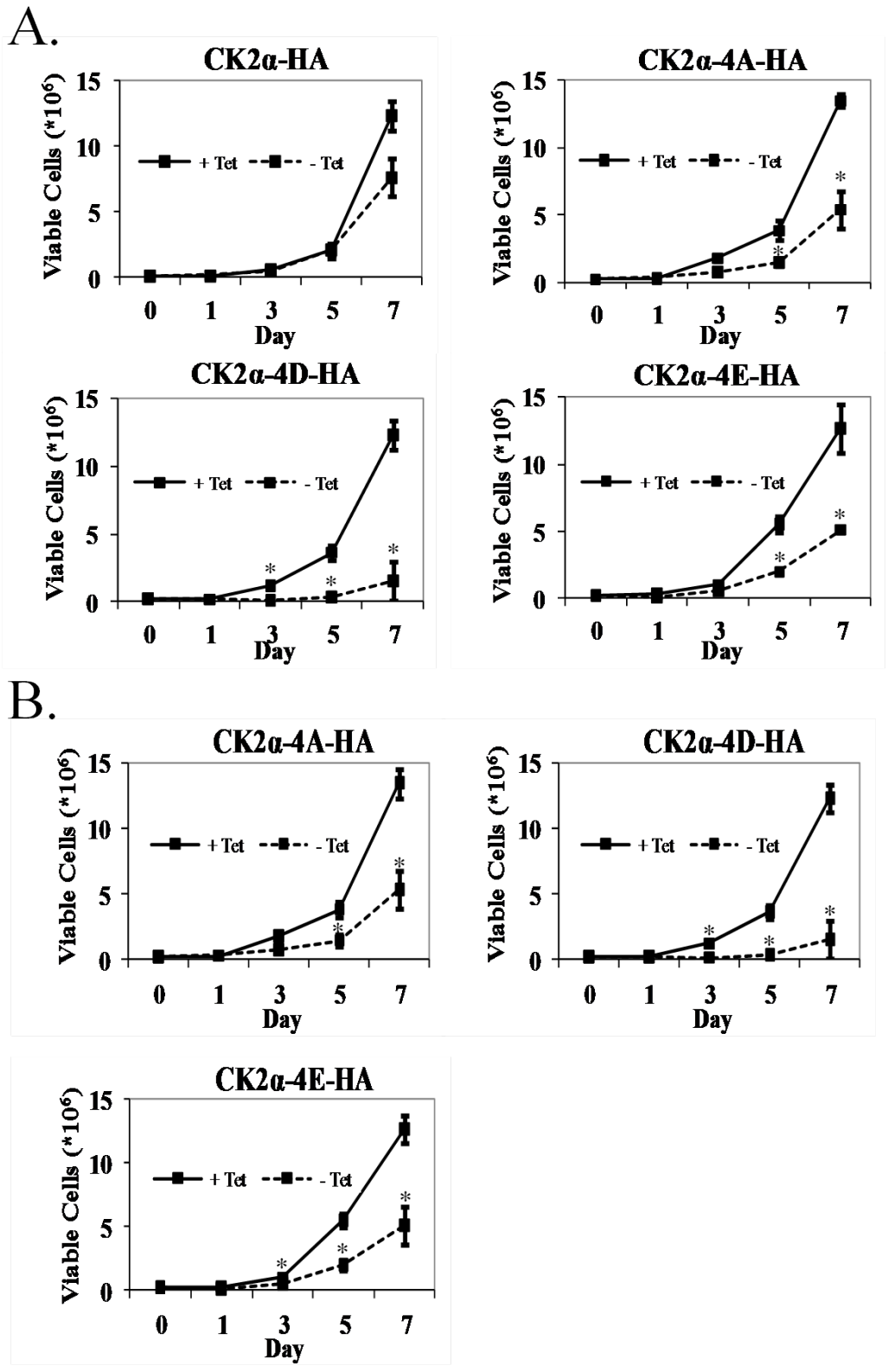
A.
CK2α-HA
CK2α-4A-HA
CK2α-4D-HA
CK2α-4E-HA
B.
CK2α-4A-HA
CK2α-4D-HA
CK2α-4E-HA
Viable Cells (*10⁶)
+ Tet
- Tet
Day
0
1
3
5
7
0
5
10
15
*

Figure 2.5 Cell proliferation profiles of inducible wild-type and mutant CK2α cell lines show decreased growth with phosphorylation-site mutant expression. A. Cells expressing CK2α-HA, CK2α-4A-HA, CK2α-4D-HA, and CK2α-4E-HA were seeded in six-well dishes at 2 x 10^4 cells/well and cultured in the presence (+ Tet, straight line) or absence (- Tet, dashed line) of 1.5 μg/μl tetracycline at Day 0. The growth media was changed every three days. Cell counts were obtained in triplicate for a period of 7 days. The results represent the average of three independent experiments. The error bars indicate one standard deviation from the mean. * indicates a significant difference between + tetracycline and – tetracycline samples ($P < 0.05$). B. Alternate cell lines expressing CK2α-4A-HA, CK2α-4D-HA, and CK2α-4E-HA were treated as in panel A.

Cells were fixed at two hour intervals for 24 hours, stained with PI, and analyzed by flow cytometry. Samples from each stable cell line without protein induction showed similar cell cycle progression profiles, with a maximum amount of cells containing 4N DNA content 8-12 hours after release from the thymidine block (Figure 2.6B). Profiles from cells expressing CK2α phosphomimetic mutants showed a maximum amount of 4N cells at slightly earlier time points, and all three phosphorylation site mutants showed a marked decrease in cells with 4N DNA content. The CK2α-4A-HA expressing cells show a modest decrease in mitotic cells at the 10 and 12 hour time points, while the CK2α-4D-HA and CK2α-4E-HA expressing cells show more marked decreases in mitotic cells from the 10-hour time point onwards. This indicates that expression of CK2α phosphorylation site mutants may affect the timing of transit to G2/M, and lead to loss of cells in mitosis. When cells were treated as in Figure 2.6A, but induced for protein expression six hours before release from thymidine block, there were no differences in cell cycle profiles compared to cells induced at the time of release (Figure 2.6C), indicating that expression of CK2α phosphorylation-site mutants does not affect S phase entry or progression.

The loss of mitotic cells observed could have several explanations, including cell cycle arrest, or loss of cells due to cell death. To investigate whether the loss of mitotic cells is due to G2 arrest, we employed Phospho-Histone H3 (Serine 10) staining and PI staining to distinguish between G2 cells and mitotic cells (39). Cells were released from a double thymidine block and protein expression was induced as in Figure 2.7A. At the 12 hour time point, cells were fixed and analyzed for Phospho-Histone H3 positivity and DNA content. Representative histograms are shown in Figure 2.7A.

Figure 2.6

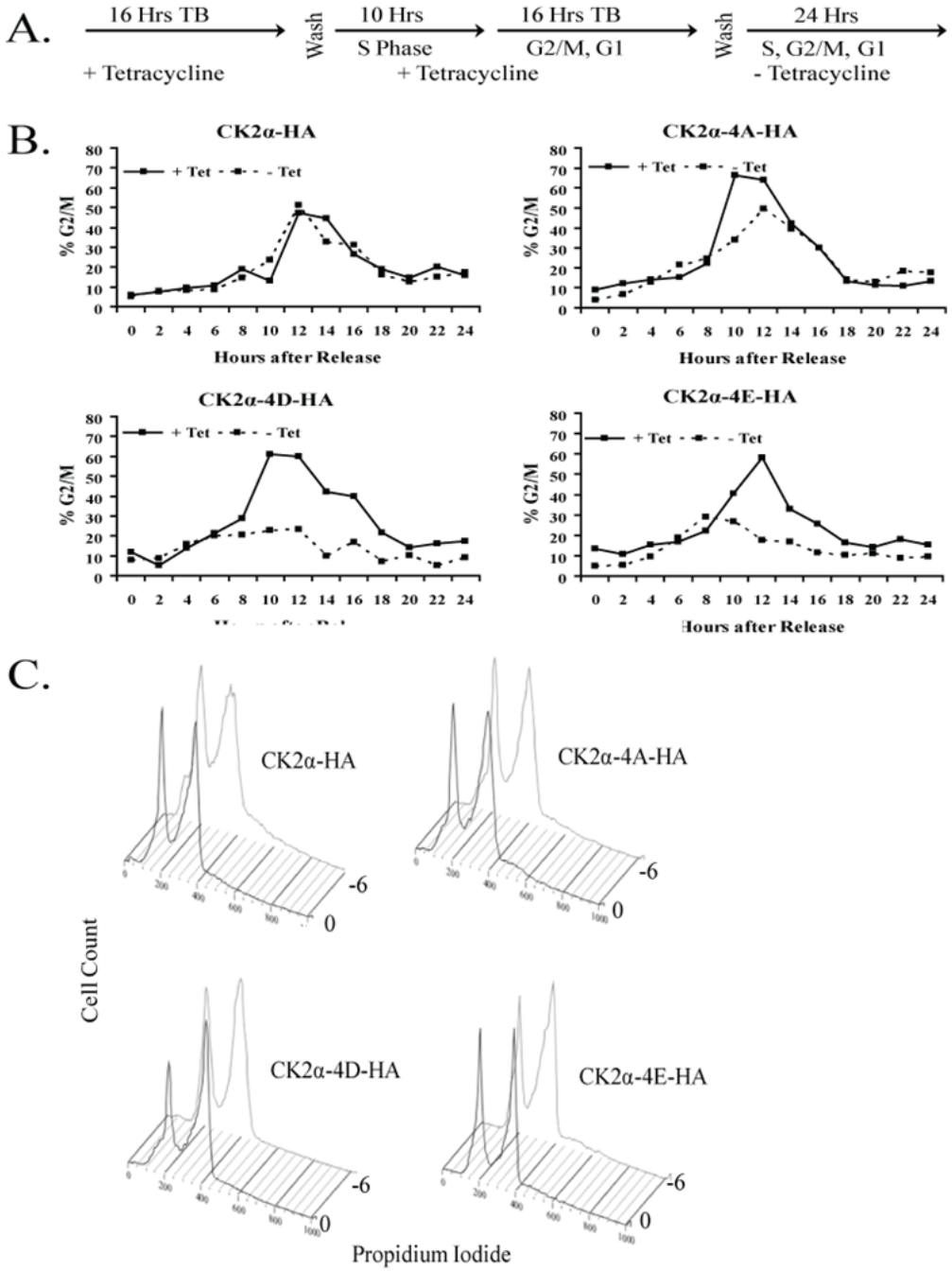
A.
16 Hrs TB
+ Tetracycline
Wash
10 Hrs
S Phase
+ Tetracycline
16 Hrs TB
G2/M, G1
Wash
24 Hrs
S, G2/M, G1
- Tetracycline
B.
CK2α-HA
CK2α-4A-HA
CK2α-4D-HA
CK2α-4E-HA
+ Tet
- Tet
% G2/M
Hours after Release
C.
CK2α-HA
CK2α-4A-HA
CK2α-4D-HA
CK2α-4E-HA
Cell Count
Propidium Iodide
-6
0

Figure 2.6 Expression of CK2α phosphomimetic mutants disrupts cell cycle progression. A. CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA cell lines were synchronized using a double thymidine block (TB), then cultured in the presence (+ tet) or absence (- tet) of tetracycline for 24 hours. B. Cell cycle profiles of synchronized CK2α cell lines. The 0 hr time point was denoted as the point in time immediately after the double thymidine block. Cells were harvested and fixed at 2 hour intervals, stained with PI and analyzed by flow cytometry. The percentage of cells in G2/M was taken from the percentage of cells containing 4N DNA content. C. Cells expressing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA were treated as in panel A, but were induced for protein expression either at the same time as release from S-phase arrest (labelled 0; trace in black) or six hours before S phase arrest (labelled -6; trace in grey). After 12 hours of cell cycle progression, cells were fixed and stained with Propidium Iodide and subjected to cell cycle analysis.

Figure 2.7

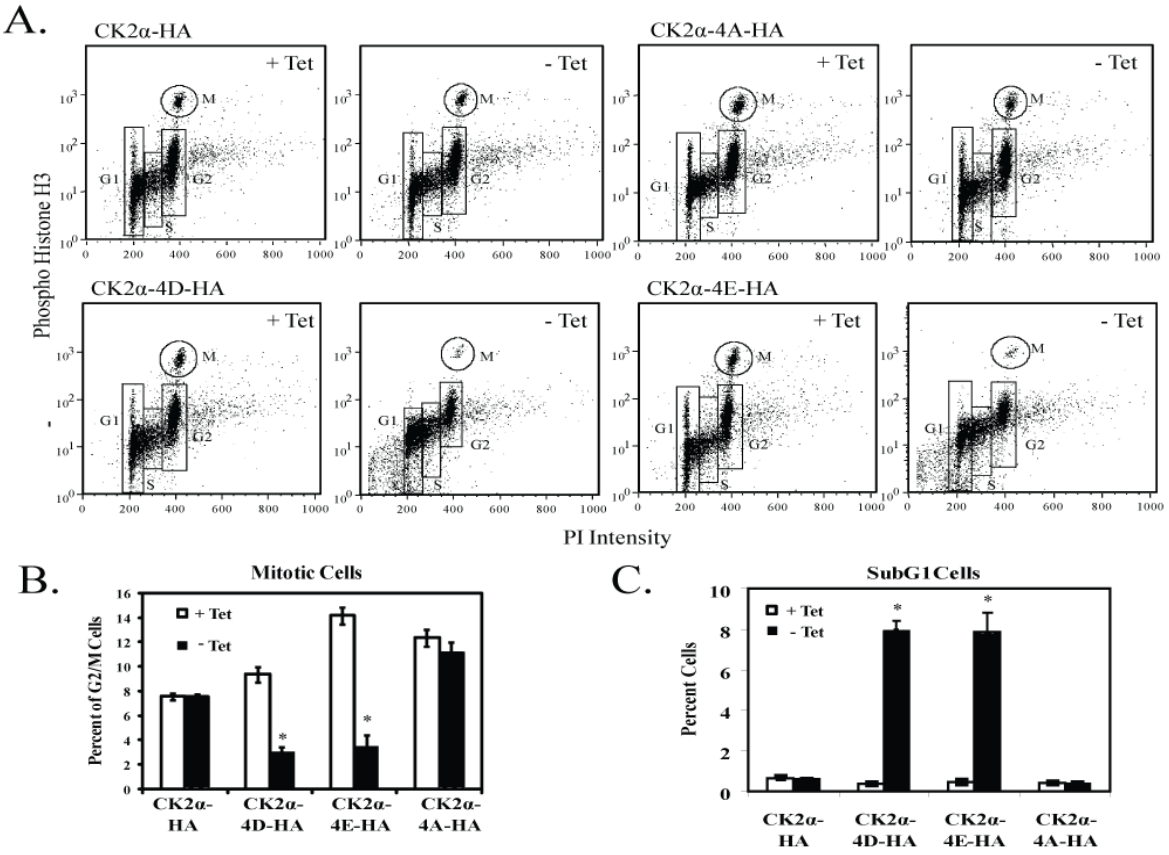

Figure 2.7 Expression of CK2α phosphomimetic mutants leads to loss of mitotic cells. A. Phospho-Histone H3 (Serine 10) staining of cells twelve hours after double thymidine block release and induction of CK2α phosphorylation site mutants. Cells were fixed and stained with Phospho-Histone H3 (Serine 10) antibody with a FITC-GAR secondary antibody and PI. Samples were analyzed by flow cytometry. B. Graphical representation of the percentage of G2/M cells in mitosis 12 hours after release from double thymidine block. C. Graphical representation of the amount of cells with Sub-G1 levels of DNA 12 hours after release from double thymidine block. For B and C, significant differences between each cell line in the presence or absence of tetracycline are denoted by * ($P > 0.05$ by pair wise analysis of variance).

The cell cycle distribution for each cell line, averaged from three independent experiments, is shown in Table 2.1. Recapitulating the results seen in Figure 2.6B, expression of CK2α-HA had no effect on the percentage of cells in mitosis. Compared to the total percentage of cells in G2/M, expression of CK2α-4D-HA and CK2α-4E-HA resulted in an almost complete loss of mitotic cells (Figure 2.7B). The decrease in mitotic cells did not accompany a corresponding increase in G2 cells, as would be expected if the loss of mitotic cells were due to a G2/M arrest. In fact, the amount of G2 cells also significantly decreases upon expression of CK2α-4D-HA or CK2α-4E-HA (Table 2.1). Interestingly, the CK2α-4D-HA and CK2α-4E-HA cell lines showed an accumulation of cells with sub-G1 levels of DNA upon expression of phosphomimetic CK2α (Figure 2.7C). No accumulation of sub-G1 cells was observed with either CK2α-HA or CK2α-4A-HA expression. Therefore, we conclude that the loss of mitotic cells observed upon expression of the CK2α phosphomimetic forms is not due to cell cycle arrest in S phase or G2 phase, but instead may be attributed to cell death.

Phosphomimetic CK2α expression causes cell death by mitotic catastrophe

Cell cycle analysis of cells expressing CK2α phosphomimetic mutants showed a dramatic increase in cells with sub-G1 amounts of DNA, indicating cell death. To quantitate the extent of cell death upon phosphomimetic CK2α expression, we arrested cells in S phase by double thymidine block and induced protein expression upon release into the cell cycle (Figure 2.8A). Cells were allowed to progress through mitosis and were stained 24 hours after thymidine release with Trypan Blue. In this assay, viable cells remain unstained, while nonviable cells are stained dark blue. While the wild type CK2α-HA cell line and the nonphosphorylatable CK2α-4A-HA cell line showed

TABLE 2.1. Cell cycle distribution of cells expressing CK2α phosphorylation site mutants 12 hours after thymidine release

	Percent Cells[c]				
Cell Line	Sub-G1	G1	S	$G2_a$	M_b
CK2α-HA					
+Tetracycline	0.64	32.04	13.77	48.79	4.01
-Tetracycline	0.59	29.89	15.16	49.60	4.13
CK2α-4D-HA					
+Tetracycline	**0.36**	28.91	17.97	**46.79**	**4.85**
-Tetracycline	**7.92**	33.21	24.98	**32.27**	**0.97**
CK2α-4E-HA					
+Tetracycline	**0.44**	31.71	15.47	**44.08**	**7.26**
-Tetracycline	**7.85**	33.05	22.77	**34.40**	**1.23**
CK2α-4A-HA					
+Tetracycline	0.41	31.22	16.36	44.67	6.80
-Tetracycline	0.35	31.29	15.45	46.59	5.91

[a] Cells with 4N DNA negative for Phospho-Histone H3 staining

[b] Cells with 4N DNA positive for Phospho-Histone H3 staining

[c] Bold font indicates significant differences between + tet and – tet samples ($P < 0.01$)

no decrease in the amount of viable cells upon CK2α expression, the CK2α-4D-HA and CK2α-4E-HA cell lines showed significant decreases in viability upon protein expression (Figure 2.8B). To ensure that the observed cell death was related to cell cycle progression and not a direct result of CK2α phosphomimetic expression, cells were induced for protein expression for 24 hours in a continuous thymidine-induced S-phase arrest. There was little evidence of cell death in CK2α phosphomimetic expressing cells arrested in S phase; therefore the cell death observed in the CK2α phosphomimetic cell lines requires both protein expression and progression through the cell cycle. Since the cell death observed was cell cycle dependent and seemed to involve the specific loss of mitotic cells, we hypothesized that the loss of mitotic cells is due to induction of mitotic catastrophe. To confirm that cells expressing CK2α-4D-HA or CK2α-4E-HA are dying during the process of cell division, we employed immunostaining to detect cells that are simultaneously mitotic and apoptotic. To identify mitotic cells, cells were immunostained with a Phospho-Histone H3 (Serine 10) antibody. We used Cytochrome C release from the mitochondria as a marker of early apoptosis, combining immunostaining with a Cytochrome C antibody with MitoTracker® Deep Red 633 FM staining to visualize the mitochondria. A representative cell in the process of both mitosis and apoptosis is shown in Figure 2.8C. As shown in Figure 2.8D, expression of CK2α-4D-HA or CK2α-4E-HA caused a large increase in mitotic catastrophe over baseline levels. Expression of neither CK2α-HA nor CK2α-4A-HA caused any increase in the amount of mitotic catastrophe observed. From this we conclude that expression of CK2α phosphomimetic mutant proteins results in cell death by mitotic catastrophe.

Figure 2.8

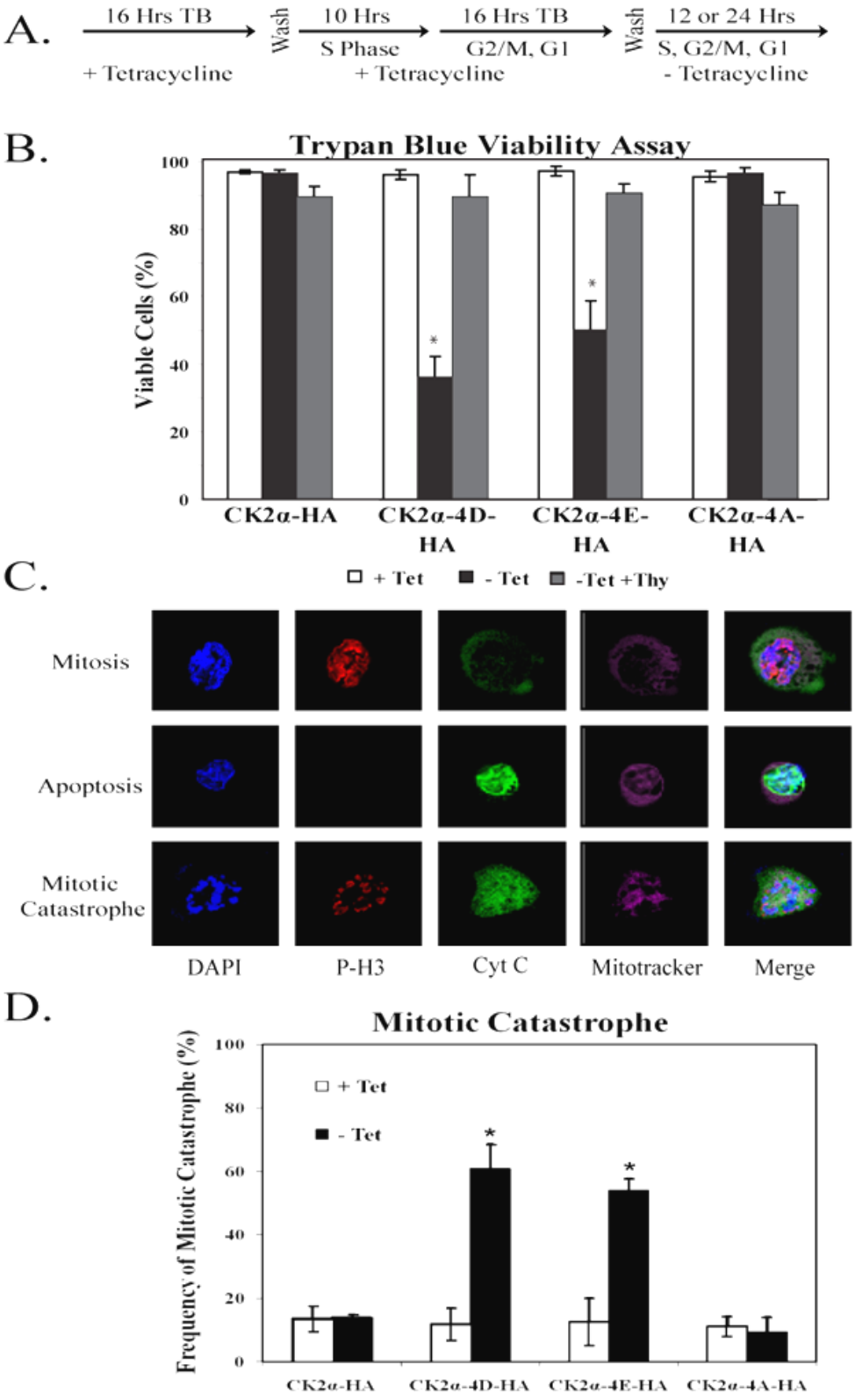
A.
16 Hrs TB
+ Tetracycline
Wash
10 Hrs
S Phase
+ Tetracycline
16 Hrs TB
G2/M, G1
Wash
12 or 24 Hrs
S, G2/M, G1
- Tetracycline
B.
Trypan Blue Viability Assay
Viable Cells (%)
100
80
60
40
20
0
*
*
CK2α-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA
+ Tet
- Tet
-Tet +Thy
C.
Mitosis
Apoptosis
Mitotic Catastrophe
DAPI
P-H3
Cyt C
Mitotracker
Merge
D.
Mitotic Catastrophe
Frequency of Mitotic Catastrophe (%)
100
80
60
40
20
0
+ Tet
- Tet
*
*
CK2α-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA

Figure 2.8 Phosphomimetic CK2α expression causes cell death by mitotic catastrophe. A. CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA cell lines were synchronized in S phase by double thymidine block (TB). Upon release, cells were induced for CK2α expression and analyzed 12 hours (Panels C and D) or 24 hours (Panel B) after release into the cell cycle. B. Cells were incubated 24 hours after release from thymidine treatment and stained with Trypan Blue to assess viability. The percentage of cells excluding blue staining (indicating cell viability) was tabulated for at least 200 cells in each of three independent experiments. Cells were also maintained in S phase by continued thymidine treatment and assayed for viability. The error bars indicate one standard deviation from the mean. Significant differences between each cell line in the presence or absence of tetracycline are denoted by * ($P > 0.05$ by pair wise analysis of variance). C. Cells expressing wild type or mutant forms of CK2α were fixed after 12 hours of cell cycle progression and immunostained with antibodies against Phospho-Histone H3 Serine 10 (P-H3), and Cytochrome C (Cyt C). Mitochondria were stained with MitoTracker® Deep Red 633 FM (Mitotracker) to visualize Cytochrome C release. D. The percentage of cells exhibiting features of mitotic catastrophe was tabulated for at least 100 mitotic cells in each of three independent experiments. The error bars indicate one standard deviation from the mean. Significant differences between each cell line in the presence or absence of tetracycline are denoted by * ($P > 0.05$ by pair wise analysis of variance).

Mitotic defects induced by CK2α phosphomimetic expression include aberrant spindle formation and missegregation of chromosomes

Expression of CK2α phosphomimetic mutants caused a dramatic increase in cell death by mitotic catastrophe. To further examine the mitotic defects caused by expression of CK2α-4D-HA or CK2α-4E-HA, cells were arrested at S phase, released into mitosis and induced for expression of CK2α phosphorylation site mutant proteins (Figure 2.9A). Cells were then fixed and immunostained with antibodies against β-Tubulin and Pericentrin, to visualize the mitotic spindle and the centrosomes respectively (5). Interestingly, cells expressing phosphomimetic forms of CK2α show multiple centrosomes, resulting in the formation of pseudobipolar, tripolar, and multipolar cells (Figure 2.9B). The aberrant numbers of centrosomes in these cells appears to be due to centrosome fragmentation and not over duplication, as cells expressing CK2α-4D-HA or CK2α-4E-HA display proper numbers of centrosomes in S phase (Data not shown). Even among the proportion of phosphomimetic CK2α-4D-HA and CK2α-4E-HA expressing cells that displayed two centrosomes, we noted abnormalities in the chromosomal segregation, including chromosomes that did not seem to line up properly at the metaphase plate, and lagging chromosomes during separation in anaphase. When these varied phenotypes were quantified, approximately half of the cells expressing CK2α-4D-HA or CK2α-4E-HA showed defects in chromosomal segregation (Figure 2.9C). The dramatic phenotypes observed upon expression of phosphomimetic CK2α indicate that CK2α phosphorylation may be involved in maintenance of centrosome integrity and proper chromosomal segregation.

Figure 2.9

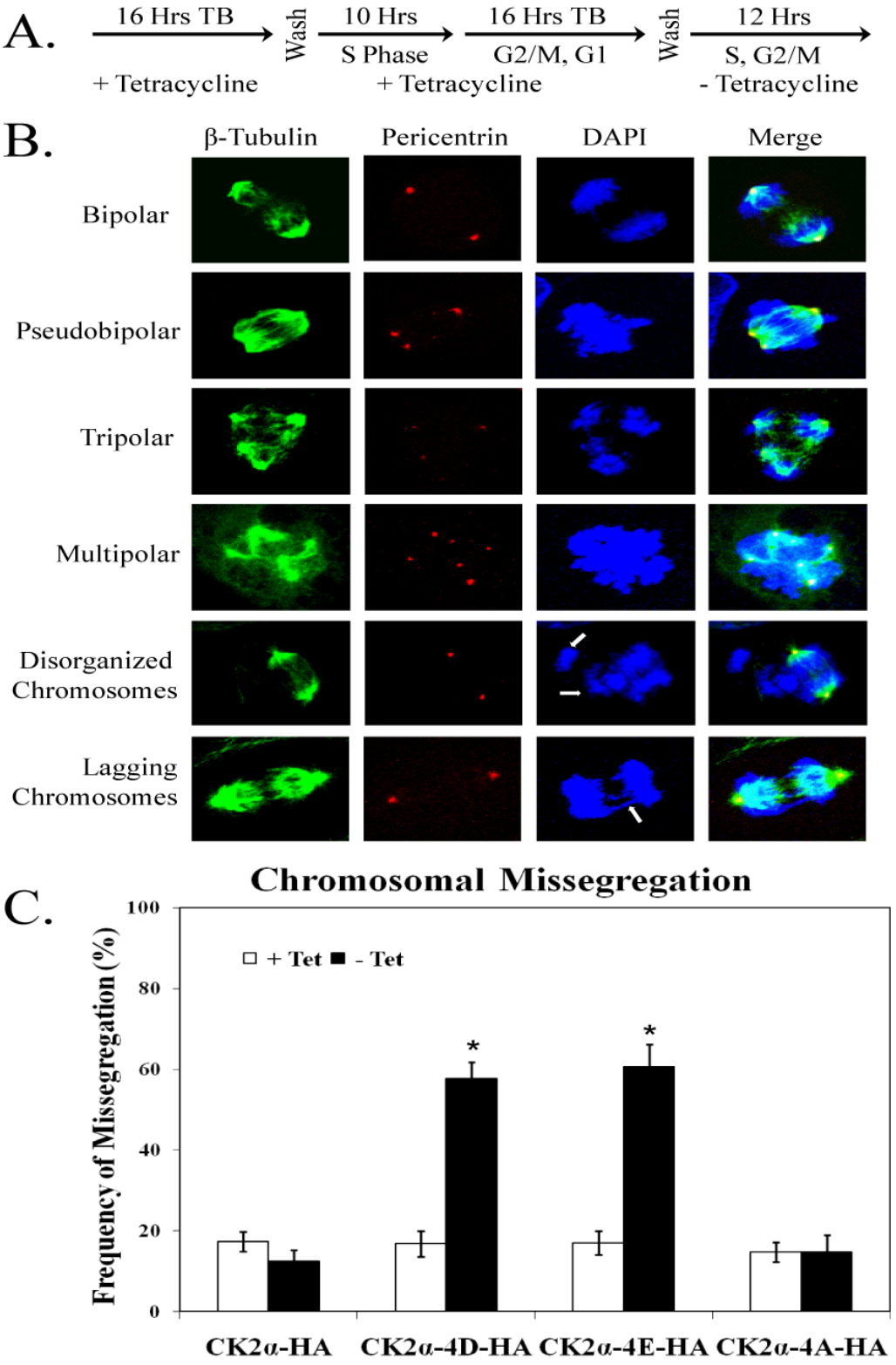
A.
16 Hrs TB
+ Tetracycline
Wash
10 Hrs
S Phase
+ Tetracycline
16 Hrs TB
G2/M, G1
Wash
12 Hrs
S, G2/M
- Tetracycline
B.
β-Tubulin
Pericentrin
DAPI
Merge
Bipolar
Pseudobipolar
Tripolar
Multipolar
Disorganized Chromosomes
Lagging Chromosomes
C.
Chromosomal Missegregation
Frequency of Missegregation (%)
100
80
60
40
20
0
□ + Tet ■ - Tet
*
*
CK2α-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA

Figure 2.9 Phosphomimetic CK2α expression results in aberrant mitotic spindle formation and missegregation of chromosomes. A. CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, and CK2α-4A-HA cell lines were synchronized in S phase by double thymidine block. Upon release, cells were induced for CK2α expression and fixed 12 hours after release into the cell cycle. B. Cells were immunostained with antibodies against Pericentrin (to visualize centrosomes) (Red) and β-Tubulin (to visualize microtubules) (Green). DNA was stained with DAPI. C. The percentage of cells with missegregation of chromosomes was tabulated for at least 100 anaphase cells in each of three independent experiments. The error bars indicate one standard deviation from the mean. Significant differences between each cell line in the presence or absence of tetracycline are denoted by * ($P > 0.05$ by pair wise analysis of variance).

Nonphosphorylatable CK2α expression abrogates the spindle assembly checkpoint

Phosphorylation of CK2α was originally identified in cells treated with nocodazole, and therefore with an activated Spindle Assembly Checkpoint (SAC) (32). Because of this, we examined whether disruption of CK2α phosphorylation would affect the ability of cells to arrest in mitosis after microtubule disruption by nocodazole treatment. Cells were treated with nocodazole in the presence or absence of CK2α phosphorylation site mutant expression. After fixation and PI staining, cell cycle analysis was employed. While expression of wild type or phosphomimetic CK2α had no effect on the ability of the cells to arrest in mitosis upon SAC activation, we observed a significant number of cells expressing CK2α-4A-HA that lost the ability to arrest after spindle damage, resulting in a decrease in G2/M cells and increases in both sub-G1 and G1 cells (Figure 2.10A). The average percentages of cells in each stage of the cell cycle for each cell line are shown in Table 2.2. To confirm the loss of metaphase arrest in cells expressing nonphosphorylatable CK2α, Cdk1 activity was investigated (37). Upon immunoprecipitation of Cdk1, cells expressing CK2α-4A-HA showed both decreased expression of Cyclin B1 and decreased phosphorylation of Histone H1, indicating that these cells have an abrogated SAC (Figure 2.10B). The average Cdk1 activity from three independent experiments is shown in Figure 2.10C. We conclude that proper phosphorylation of CK2α is required to maintain the SAC in response to spindle damage.

Figure 2.10

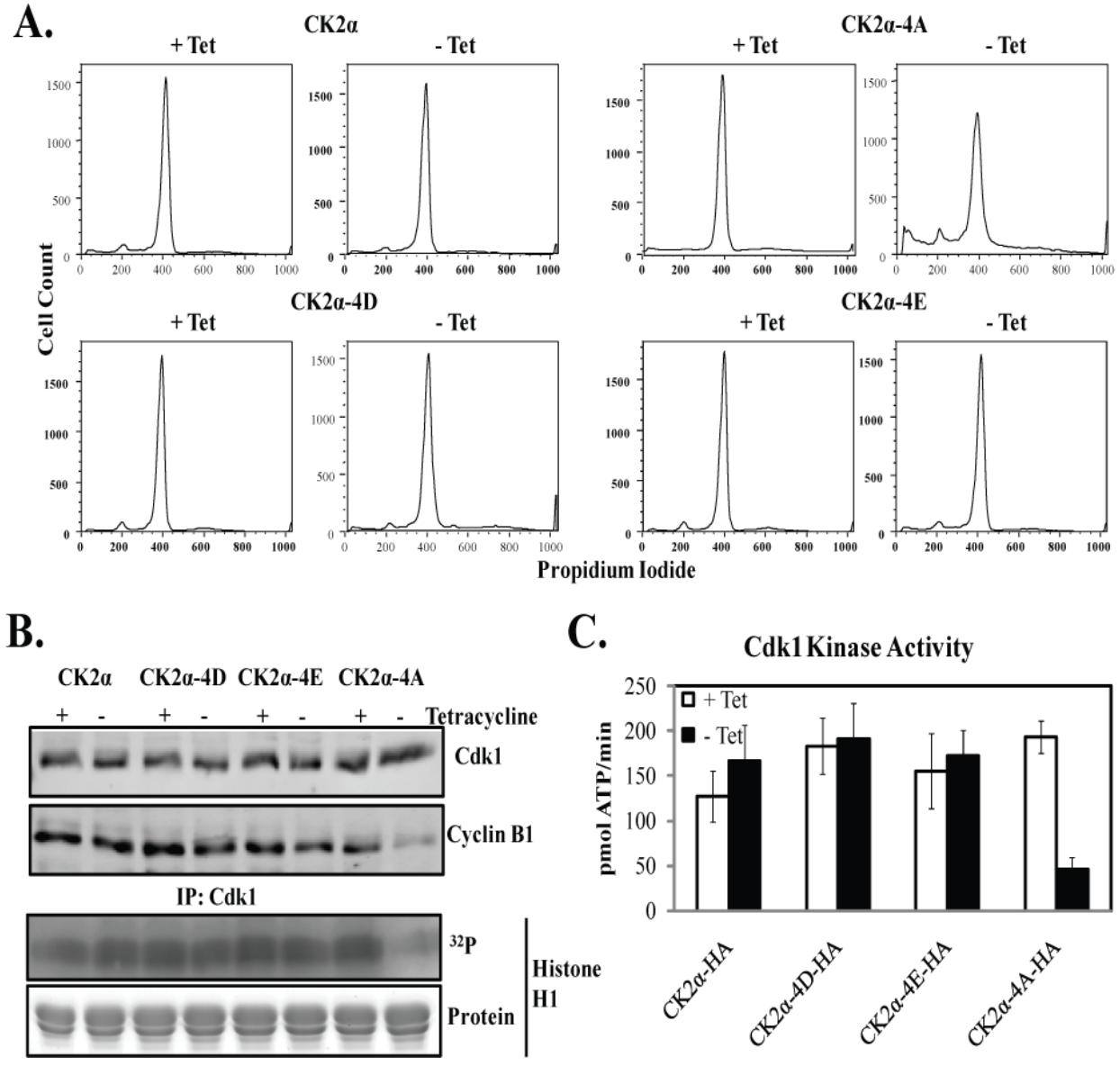
A.
CK2α
+ Tet
- Tet
CK2α-4A
CK2α-4D
CK2α-4E
Cell Count
Propidium Iodide
B.
CK2α
CK2α-4D
CK2α-4E
CK2α-4A
Tetracycline
Cdk1
Cyclin B1
IP: Cdk1
32P
Histone H1
Protein
C.
Cdk1 Kinase Activity
+ Tet
- Tet
pmol ATP/min
CK2α-HA
CK2α-4D-HA
CK2α-4E-HA
CK2α-4A-HA

Figure 2.10 Expression of non-phosphorylatable CK2α abrogates the spindle assembly checkpoint. A. Cell cycle profiles of cells expressing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, or CK2α-4A-HA were arrested in mitosis by nocodazole treatment in the presence or absence of tetracycline, followed by fixation and PI staining. Samples were analyzed by flow cytometry. B. Cdk1 was immunoprecipitated from lysates prepared from nocodazole-arrested cells expressing CK2α-HA, CK2α-4D-HA, CK2α-4E-HA, or CK2α-4A-HA. Immunoprecipitates were immunoblotted for Cdk1 and Cyclin B1, or used in kinase assays with Histone H1 and γ-^{32}P-ATP. Phosphorylation is shown by a representative autoradiograph. Total Histone H1 is shown by Coomassie Blue staining. C. Graphical representation of Cdk1 kinase activity. Results are the average of three independent experiments. Error bars indicate one standard deviation from the mean.

TABLE 2.2 Cell cycle distribution of nocodazole-arrested cells expressing CK2α phosphorylation-site mutants

	Percent Cells[a]			
Cell Line	Sub-G1	G1	S	G2/M
CK2α-HA				
+Tetracycline	7.00	6.33	10.57	88.26
-Tetracycline	7.01	6.04	11.50	73.50
CK2α-4D-HA				
+Tetracycline	3.89	3.13	10.88	71.80
-Tetracycline	4.28	7.33	17.00	63.29
CK2α-4E-HA				
+Tetracycline	3.40	5.81	11.41	76.86
-Tetracycline	4.92	8.91	18.84	73.59
CK2α-4A-HA				
+Tetracycline	**5.24**	5.57	12.37	**82.64**
-Tetracycline	**14.87**	10.93	24.74	**51.41**

[a] Bold font indicates significant differences between + tet and – tet samples ($P < 0.01$)

2.4 Discussion

In this study, we have investigated the role of CK2α phosphorylation in mitosis. Our data indicate that precise regulation of these phosphorylation events is required for proper mitotic progression. Through generation of phosphospecific antibodies against four phosphorylation sites known to be phosphorylated in nocodazole arrested cells (4, 20), we show that these sites are also phosphorylated in cells progressing through normal mitosis. Mitotic phosphorylation is strongest during prophase and metaphase and decreases during anaphase, becoming undetectable by telophase and cytokinesis. The temporal pattern of phosphorylation observed matches the temporal activation of Cdk1, the kinase believed responsible for these phosphorylation events (20).

We next sought to determine the function of CK2α phosphorylation by observation of phenotypes associated with expression of either CK2α phosphomimetic mutants (CK2α-4D-HA and CK2α-4E-HA) or non-phosphorylatable mutant CK2α (CK2α-4A-HA). Proliferation curves showed decreased growth in all three lines compared with expression of CK2α-HA, with particular defects shown in the phosphomimetic cell lines. Subsequent cell cycle analysis showed sub-G1 cells upon CK2α phosphomimetic expression, indicating that mitotic cells were dying in a cell cycle dependent manner. Upon CK2α-4D-HA or CK2α-4E-HA expression, mitotic cells undergo mitotic catastrophe, a type of cell death occurring in mitosis due to deficiency in cell cycle checkpoint control or cellular damage (6).This may serve as a mechanism to eliminate cells that, if permitted to progress through mitosis, would result in aneuploid daughter cells. The stimulus for induction of mitotic catastrophe may be the centrosomal amplification observed in phosphomimetic-expressing cells, leading to aberrant spindle

formation and chromosomal missegregation. Similar results have recently been observed in *Drosophila melanogaster*, as RNA interference silencing of the *CkIIα* gene causes mitotic abnormalities, including centrosome abnormalities and lagging chromatids (3). In mammalian cells, CK2 has been shown to colocalize with the centrosomes (9, 26) and mitotic spindle (17, 42), and many components of the mitotic machinery interact with and/or are substrates of CK2, including β-Tubulin, Microtubule-associated Proteins 1A and 1B, Tau, Condensin, and Protein Phosphatase 2A (2, 10, 14, 35). Recent proteomic investigations have identified CK2 as a component of both the centrosome and the spindle midbody (1, 34). Phosphoproteomic analysis of mitotic spindles recently identified a number of possible CK2 substrates, including both known CK2 substrates such as Topoisomerase IIα and HSP-90, and novel substrates such as Septin-2, INCENP, and MAP7 (28). While the mechanism by which aberrant CK2α phosphorylation leads to centrosomal defects is unknown, it is clear that proper regulation ensures spindle organization and maintains genomic integrity.

As CK2α phosphorylation occurs mainly during prophase and metaphase, we theorize that the defects seen upon expression of CK2α phosphorylation-site mutant proteins may be due to loss of temporal control of phosphorylation. During a normal cell division, CK2α remains unphosphorylated until the onset of prophase, when phosphorylation occurs. In the presence of spindle abnormalities, CK2α phosphorylation is at a maximum. Once all chromosomes are lined up at the metaphase plate, CK2α is dephosphorylated as the cell enters anaphase, and remains dephosphorylated until the next cell division. Defects seen upon expression of CK2α phosphorylation-site mutant proteins may represent the consequences of loss of temporal regulation of

phosphorylation. When CK2α seems phosphorylated before the onset of prophase, decreased centrosome integrity leads to abnormal spindle formation and chromosomal missegregation. Cells expressing phosphomimetic CK2α also undergo mitotic catastrophe, either as a result of these spindle abnormalities, or alternatively, when CK2α seems to remain phosphorylated after the metaphase-anaphase transition. Expression of non-phosphorylatable CK2α results in loss of cell cycle arrest after spindle damage, indicating that CK2α phosphorylation plays a role in maintaining the SAC. This result was particularly interesting. Cells expressing CK2α-4A-HA did not display dramatic mitotic defects, showing only a slight decrease in proliferation compared to cells expressing wild type CK2α. This corresponds well with the finding that not all CK2α seems to be phosphorylated in a normally dividing cell, and indicates that only a subset of CK2α needs to be modified to fulfill its mitotic purposes. However, upon treatment of cells with nocodazole, when CK2α phosphorylation is at maximum levels, expression of non-phosphorylatable CK2α neutralizes the activated SAC, allowing cells to progress through mitosis even in the presence of endogenous phosphorylated CK2α. The involvement of CK2 in SAC signaling has been previously demonstrated, as depletion of CK2 activity compromises SAC arrest after nocodazole treatment (33). However, the role of CK2α phosphorylation has not been examined.

Interestingly, expression of CK2α phosphorylation-site mutants elicits mitotic phenotypes even in the presence of comparable amounts of endogenous CK2α. Since the mutant proteins are capable of forming complexes with Myc-CK2β and presumably incorporate into mixed tetramers with endogenous CK2 subunits, this suggests that CK2α phosphorylation-site mutants may have a dominant effect over endogenous, and

presumably phosphorylated, CK2α. This dominant effect may be mediated through blocking or encouraging phosphorylation-dependent interactions between CK2 and mitotic proteins. It seems likely that the CK2α phosphorylation sites serve as a regulatory mechanism, likely through forming an interaction site for the binding of other proteins. This has been shown to be the case for Pin1, a cis/trans peptidyl-prolyl isomerase with a number of mitotic substrates. Pin1 selectively isomerizes proline residues adjacent to phosphorylated serine or threonine residues (41). While C-terminal phosphorylation of CK2α does not affect general kinase activity of CK2, as measured by kinase assays with a substrate peptide, it is plausible that phosphorylation at these sites may be important in the regulation of CK2 activity against particular substrates, as is the case for Topoisomerase IIα. The modulation of CK2 kinase activity towards Topoisomerase IIα is dependent on Pin1 binding, as a complex is formed between CK2, Pin1, and Topoisomerase IIα (27). It remains unknown whether this regulatory mechanism is involved in selective binding and modulation of additional CK2 substrates. Recently, a proteomic screen of Plk1 Polo-Box Domain (PBD) binding proteins identified CK2α as a phosphorylation-dependent mitotic binding partner for Plk1 (24). Plk1 is a mitotic kinase with multiple roles in mitotic progression (36). Interestingly, the protein sequence surrounding the CK2α T344 phosphorylation site corresponds to the consensus sequence for Plk1 PBD binding (8). Further investigation of the interplay between these two mitotic kinases may reveal precise roles for CK2α phosphorylation in mitosis.

Overall, we have shown that CK2α is phosphorylated during mitotic progression, and that these phosphorylation events do indeed have a regulatory role in the process of cell division. Substitution of these residues leads to mitotic catastrophe, defects in

centrosome amplification, and abrogation of the SAC. Collectively, these results offer evidence of a role for reversible phosphorylation of CK2α in the control of cell division.

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Chapter 3: Localization of Phosphorylated CK2α to the Mitotic Spindle is Dependent on the Peptidyl-prolyl Isomerase Pin1[1]

3.1 Introduction - Reversible phosphorylation by protein kinases is a regulatory mechanism crucial for cell signalling, providing a temporary switch in protein function leading to the proper cellular response to a stimulus. When the stimulus induces proliferation, the cell undergoes a highly regulated series of events ultimately leading to mitosis. Protein kinases play crucial roles in regulating mitotic progression, and understanding these roles will increase our understanding of the process of mitosis, and may lead to the development of targeted therapies against proliferative diseases such as cancer. Our focus is on determining the role of protein kinase CK2 in mitotic regulation. CK2 is a serine/threonine kinase with a plethora of substrates that is ubiquitously expressed and exhibits constitutive activity. CK2 is typically found as a tetramer, composed of two regulatory CK2β subunits, and two catalytic subunits, CK2α and CK2α'. CK2α and CK2α' share 90% sequence identity, differing only in their C-termini (18). Unlike many protein kinases, CK2 is not subjected to strict on/off regulation, but instead seems to be regulated through a variety of mechanisms, including subcellular localization, protein-protein interactions, and phosphorylation (27). CK2 is involved in many cellular responses, including regulation of transcription and translation, cell proliferation, and cell survival (35).

CK2 has long been recognized for its importance in mitosis. CK2 is required for progression through the G1/S and G2/M transitions in yeast and mammalian cells (9, 14, 15, 29). RNA interference silencing of the *Drosophila melanogaster CkIIα* gene leads to

[1] A modified version of this chapter is currently in revision: St-Denis NA, Bailey ML, Parker EL, Litchfield DW (2010). **Localization of CK2α to the mitotic spindle is dependent on the peptidyl-prolyl isomerase Pin1**. (Journal of Cell Science)

mitotic abnormalities (5). Furthermore, CK2 colocalizes with centrosomes and the mitotic spindle (10, 43), where it interacts with and/or phosphorylates a variety of spindle components, including β-Tubulin (8), Condensin (37), Tau (2), and Microtubule Associated Proteins 1A and 1B (2). Proteomic studies have confirmed CK2 as a component of both the centrosome and the spindle midbody (1, 33), and phosphoproteomic analyses of the mitotic spindle have identified a number of potential CK2 substrates (22, 26). Interestingly, the CK2α isoform of CK2 is maximally phosphorylated in mitosis (12) at four sites in its unique C-terminus (T344, T360, S362, and S370) (7, 13). In our previous work, we used phosphospecific antibodies to show that phosphorylation is temporally regulated, occurring during prophase and metaphase. Stable expression of phosphorylation site mutants of CK2α results in multiple mitotic defects, including centrosome amplification, chromosomal missegregation and mitotic catastrophe (34). These results indicate that proper phosphorylation of CK2α is required for mitotic progression.

Since CK2α phosphorylation does not directly affect its kinase activity (6), we investigated whether phosphorylation affects its subcellular localization during mitosis. In this report we show that phosphorylated, but not unphosphorylated, CK2α localizes to the mitotic spindle. Furthermore, we show that Pin1, a peptidyl-prolyl isomerase important for mitotic progression, is required for localization of phospho-CK2α. We conclude that CK2α phosphorylation acts as a regulatory mechanism to localize CK2 to the mitotic spindle, which may target it towards specific mitotic substrates.

3.2 Materials and Methods

Antibodies- CK2α, CK2α', and phospho-CK2α antibodies have been previously described (12, 34). The CK2α antibody used to detect non-phosphorylated CK2α (C-18) was obtained from Santa Cruz Biotechnologies. Monoclonal β-Tubulin antibodies were a gift of Dr. Lina Dagnino (Department of Physiology and Pharmacology, University of Western Ontario). Other antibodies used were: biotin-conjugated HA (3F10) (Roche), Alexa-Fluor 488-conjugated HA (Molecular Probes), DsRed (Clontech), Pericentrin (Abcam), Pin1 (R&D Systems), monoclonal Flag M2 (Sigma), polyclonal Flag M2 (Cell Signalling Technologies), and β-Actin (Sigma). Secondary antibodies were obtained from LiCOR Biosciences and Molecular Probes.

Plasmid Constructs - HA-CK2α, HA-CK2α', HA-CK2α/α', and HA-CK2α'/α constructs have been previously described (28). The unique C-terminus of CK2α was introduced into the DsRed2-C1 vector (Clontech) by PCR amplification using the forward primer 5'AATCCGCGGTACTGGACAAACTGC3' and the reverse primer 5'AAAGGATCCCATCAGGAGACAG3'. The resultant fragment was subcloned into DsRed2-C1 using SacII and BamH1 restriction sites. Alanine and aspartic acid mutations were introduced by site-directed mutagenesis using a Quik-Change II Site-Directed Mutagenesis kit (Stratagene). Short hairpin RNA (shRNA) constructs targeting Luciferase and Pin1 were obtained from M. Golding and G. DiMattia. Two Pin1 shRNAs were constructed, Pin1A (5'CACCGTCACACAGTATTTA3') and Pin1B (5'GGCCGAATTGTTTCTAGTT3'), targeting two different sequences in the 3' untranslated region of Pin1. The Luciferase shRNA has been previously described (38). For Flag-Pin1 construction, oligonucleotides containing the Flag epitope and Not1 and

EcoR1 restriction sites (5'AATTGGCAATGGACTACAAAGACGATGACGACAAG GCGGCCGCCTCAGAATTCCT3';5'CTAGAGGAATTCTGAGGCGGCCGCCTTGTC GTCATCGTCTTTGTAGTCCATTGCC3') were annealed and introduced into pcDNA3.1. Pin1, Pin1-Y23A, Pin1-R68/69A, and Pin1-C113S were subcloned from previously described pY204 plasmids (4) using Not1 and EcoR1 and ligated into pcDNA3.1-Flag. All sequences were confirmed by DNA sequencing.

Cell Culture- U2OS and HeLa cells were cultured in Dulbecco's modified eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotic supplements (0.1 mg/mL streptomycin and 100 U/mL penicillin; Life Technologies Inc.). Cells were synchronized in S phase by two 16 hour treatments with 2 mM thymidine (Sigma) separated by 10 hours incubation without thymidine. To synchronize cells in G2/M, cells were released from S-phase arrest and treated with 40 ng/mL nocodazole (Sigma) for 18 hours. Plasmid constructs were introduced into cells by calcium phosphate transfection. Cell lines stably expressing shRNA plasmids targeting Luciferase or Pin1 were generated by addition of 4 μg/ml puromycin (Sigma) 48 hours post-transfection. Discrete colonies formed were then isolated and expanded. Samples from individual colonies were screened for Pin1 levels by immunoblot.

Cell lysis and phosphatase treatment- Cells were lysed on ice in NP-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 1 μg/mL each Pepstatin A and leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, and 4 mM Na_2VO_4). Following sonication on ice, lysates were centrifuged at 13000 X g for 15 minutes at 4°C. Dephosphorylation of lysates was performed using λ-phosphatase (New England Biolabs). Cells were lysed in the absence of phosphatase inhibitors, and incubated 30 min

at 37°C in the presence of 1 mM $MnCl_2$, 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, and 200 U of λ-phosphatase.

Immunoblot Analysis- The protein concentration of each sample was determined with the BCA protein assay (Pierce). Equal amounts of cell lysates were separated by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (11). Proteins were then transferred to nitrocellulose membrane for 1 hour at 15 V and 0.3 A using a Trans-Blot semidry electrophoretic transfer apparatus (Bio-Rad). Membranes were blocked for one hour with 5% bovine serum albumin in Tris-buffered saline before overnight incubation with the primary antibody. After one hour incubation with fluorophore-linked secondary antibodies, immune complexes were detected on a LiCOR near-infrared fluorescent scanner using Odyssey V3.0 software.

Immunostaining and Microscopy- Cells used for immunostaining experiments were grown on poly-L-lysine coated coverslips (BD Falcon). Cells were fixed in 3.7% paraformaldehyde for 30 min before incubation with 0.1% Glycine in PBS for 30 min, permeabilization with 0.1% Triton X-100 in PBS for 5 min, and blocking with 5% FBS for 1 hour. Primary and secondary antibody incubations were done for 1 hour and 30 min respectively, followed by staining with 4',6'-diamidino-2-phenylindole (DAPI). All incubations were performed at 37°C. Coverslips were mounted on slides with AirVol. Microscopic images were acquired with Zeiss Zen software on a Zeiss META 510 LSM confocal microscope. Images were processed using Adobe Photoshop.

Mitotic Spindle Isolation- Mitotic spindles were isolated from U2OS cells by the method of Silljé and Nigg (32). Briefly, cells were arrested in G1/S with 1.6 μg/mL aphidicolin

(Sigma) for 16 hours, released into the cell cycle for 8 hours, and then arrested in mitosis by incubation with 40 ng/mL nocodazole for 16 hours. Mitotic cells were collected and washed to remove nocodazole, then reincubated until the majority of cells entered metaphase, at which point the cells were treated with 5 μg/mL Paclitaxel (Taxol; Calbiochem). Cells were collected, washed with PBS supplemented with 2 ug/mL Latrunculin B, 1 mM PMSF, and 5 μg/mL Taxol, then lysed with spindle lysis buffer (100 mM PIPES pH 6.9, 1 mM Mg2SO4, 2 mM EGTA, 0.5% NP-40, 5 μg/mL Taxol, 2 μg/mL Latrunculin B, 200 μg/mL DNAse1, 10 μg/mL RNAseA, 1 U/mL micrococcal nuclease, 20 U/mL Benzonase, 1 mM PMSF, 1 μg/mL of each of pepstatin A, leupeptin, and aprotinin, and 20 mM β-glycerophosphate). Following incubation with 1 mM PIPES pH 6.9 with 5 μg/mL Taxol, isolated spindles were confirmed by phase microscopy and resuspended in Laemmli buffer for SDS-PAGE.

Dephosphorylation Assay- Phosphorylated CK2α was immunoprecipitated from nocodazole arrested Pin1 shRNA expressing cells using Protein A Sepharose and 1 μL of anti-CK2α serum for 1 h at 4°C, then incubated for 10 min at 30°C in the presence of 1 nM, 10 nM, or 100 nM GST, GST-Pin1, or GST-Pin1-R68/69A, purified as previously described (3), in 20 mM HEPES pH 7.0 with 1 mM DTT, 1 mM $MnCl_2$, 10 ug/mL BSA, and 50 mM Leupeptin. 0.05 U of PP2A (Millipore) was added for 30 min at 30°C. Okadiac acid was used at 10 nM. Reactions were stopped by addition of Laemmli buffer. The assay was performed in triplicate.

3.3 Results

Phosphorylated CK2α localizes to the mitotic spindle

CK2α has been previously reported to localize to the mitotic spindle (10, 43), but the phosphorylation-dependence of this localization has not been examined. To investigate this, we used phosphospecific antibodies targeted against the T344, T360/S362, and S370 phosphorylation sites (34). These antibodies specifically detected phosphorylated CK2α in nocodazole-arrested cells, but showed no reactivity against unphosphorylated CK2α in thymidine-blocked cells (Figure 3.1A, the S370 antibody is shown). Antibody phosphospecificity was confirmed using λ phosphatase treatment of mitotic lysates (Figure 3.1B). To detect non-phosphorylated CK2α, we used an antibody that was serendipitously found to detect only non-phosphorylated CK2α, presumably due to its generation against an overlapping and non-phosphorylated CK2α C-terminal epitope (Figure 3.1A,B). Mitotic cells were immunostained for β-Tubulin and either phospho-CK2α or non-phospho-CK2α, and confocal microscopy was used to examine localization (Figure 3.1C). In all three cases, phospho-CK2α colocalized with β-Tubulin, while the non-phospho-CK2α did not. To extend this result to a population of cells, we isolated mitotic spindles from cells and immunoblotted the spindles for CK2α (Figure 3.1D). Whole mitotic lysates are also shown. Cells treated with nocodazole, which inhibits mitotic spindle formation by inducing microtubule depolymerisation (44), were used as a negative control. Confirming the colocalization results, phospho-CK2α copurified with isolated mitotic spindles, while non-phospho-CK2α did not. These results indicate that phosphorylated CK2α preferentially localizes to the mitotic spindle.

Figure 3.1

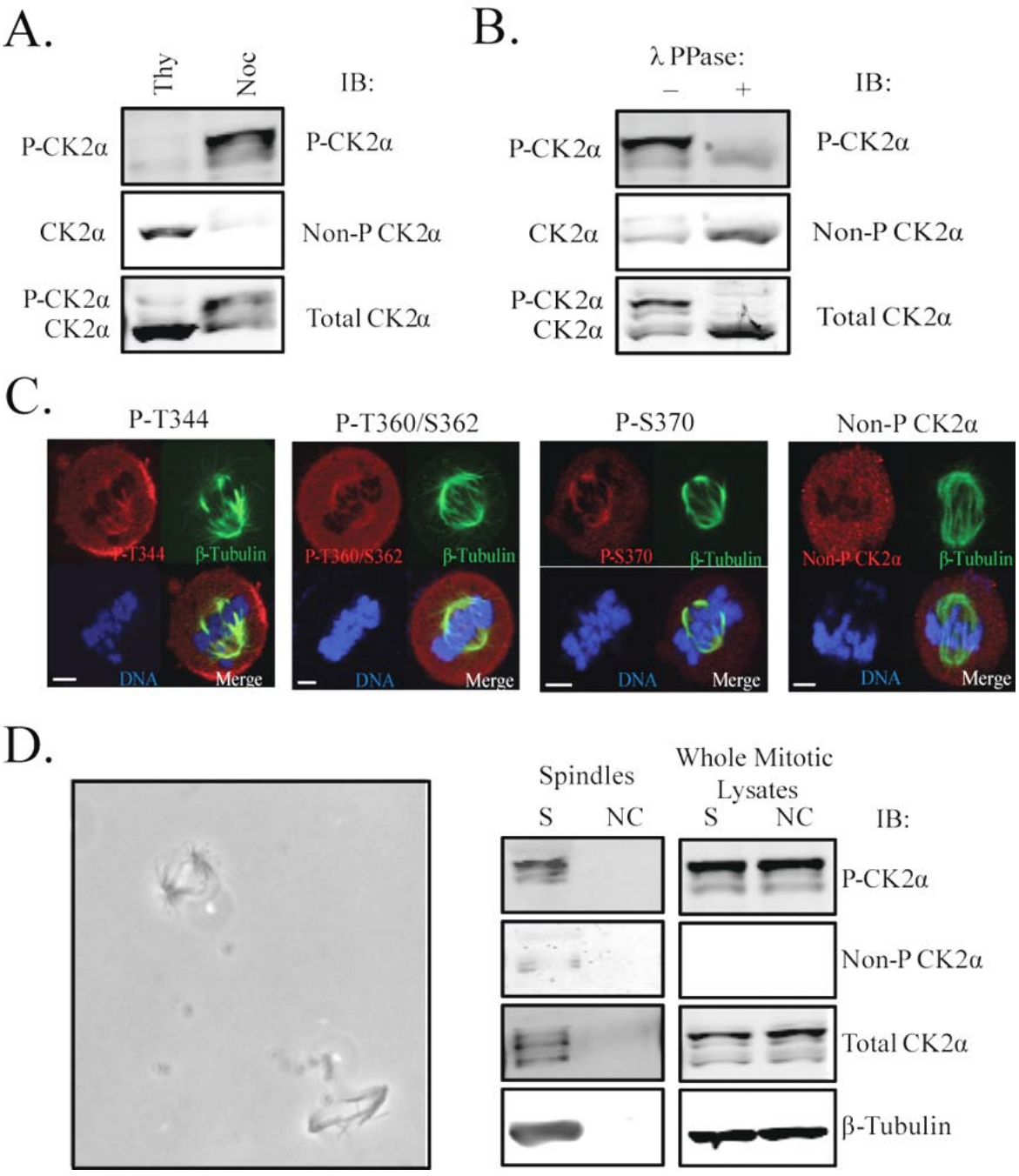

A.
Thy
Noc
IB:
P-CK2α
CK2α
P-CK2α
CK2α
P-CK2α
Non-P CK2α
Total CK2α
B.
λ PPase:
–
+
IB:
P-CK2α
CK2α
P-CK2α
CK2α
P-CK2α
Non-P CK2α
Total CK2α
C.
P-T344
P-T360/S362
P-S370
Non-P CK2α
P-T344
β-Tubulin
P-T360/S362
β-Tubulin
P-S370
β-Tubulin
Non-P CK2α
β-Tubulin
DNA
Merge
DNA
Merge
DNA
Merge
DNA
Merge
D.
Spindles
Whole Mitotic Lysates
S
NC
S
NC
IB:
P-CK2α
Non-P CK2α
Total CK2α
β-Tubulin

Figure 3.1 Phosphorylated CK2α localizes to the mitotic spindle. (A) Lysates from cells arrested in S phase with Thymidine (Thy) or prometaphase with Nocodazole (Noc) were immunoblotted with antibodies targeting phosphorylated CK2α, nonphosphorylated CK2α, and total CK2α. (B) Nocodazole-arrested lysates were also dephosphorylated with λ Phosphatase before immunoblotting as in A. (C) Mitotic cells were immunostained with β-Tubulin and either phospho-CK2α or non-phospho-CK2α antibodies, followed by confocal microscopy. Scale bars, 5 μm. (D) Left panel: Purified mitotic spindles visualized by phase microscopy. Right panel: Purified mitotic spindles were immunoblotted with phospho-CK2α, non-phospho-CK2α, and total-CK2α antibodies. β-Tubulin immunoblotting shows the presence of mitotic spindles. S, spindles; NC, nocodazole control.

To confirm the specificity of the phosphospecific antibodies, peptide competition assays were performed, in which each antibody was preincubated with either non-phosphorylated or phosphorylated versions of the peptide epitope used to generate the antibody. While incubation with non-phosphorylated peptide did not affect spindle localization, incubation with phosphorylated peptide caused an almost complete loss of reactivity, confirming that the phosphospecific antibodies are detecting phospho-CK2α on the mitotic spindle (Figure 3.2).

Spindle localization requires phosphorylation of the unique C-terminus of CK2α

Since the only major difference between the two catalytic isoforms of CK2 is the presence of the extended C-terminal tail on CK2α, which includes the mitotic phosphorylation sites (18), we next examined whether interchanging the C-termini of the two isoforms would affect their mitotic localization. Plasmids expressing HA-tagged CK2α and CK2α', as well as chimeric versions with the C-termini interchanged (HA-CK2α/α', HA-CK2α'/α) (28) were transfected into U2OS cells (Figure 3.3A, and immunoblot analysis showed uniform expression (Figure 3.3B). Immunoblotting lysates from mitotic cells expressing these plasmids for phospho-CK2α showed that the HA-CK2α'/α protein is phosphorylated at levels similar to HA-CK2α, while neither HA-CK2α' or HA-CK2α/α' were phosphorylated (Figure 3.3C). HA-CK2α co-purified with mitotic spindles, while very little HA-CK2α' was detected (Figure 3.3D). It seems that spindle localization requires the CK2α C-terminus, as when the CK2α C-terminus was replaced with the CK2α' C-terminus (HA-CK2α/α'), the chimeric protein copurified with

Figure 3.2

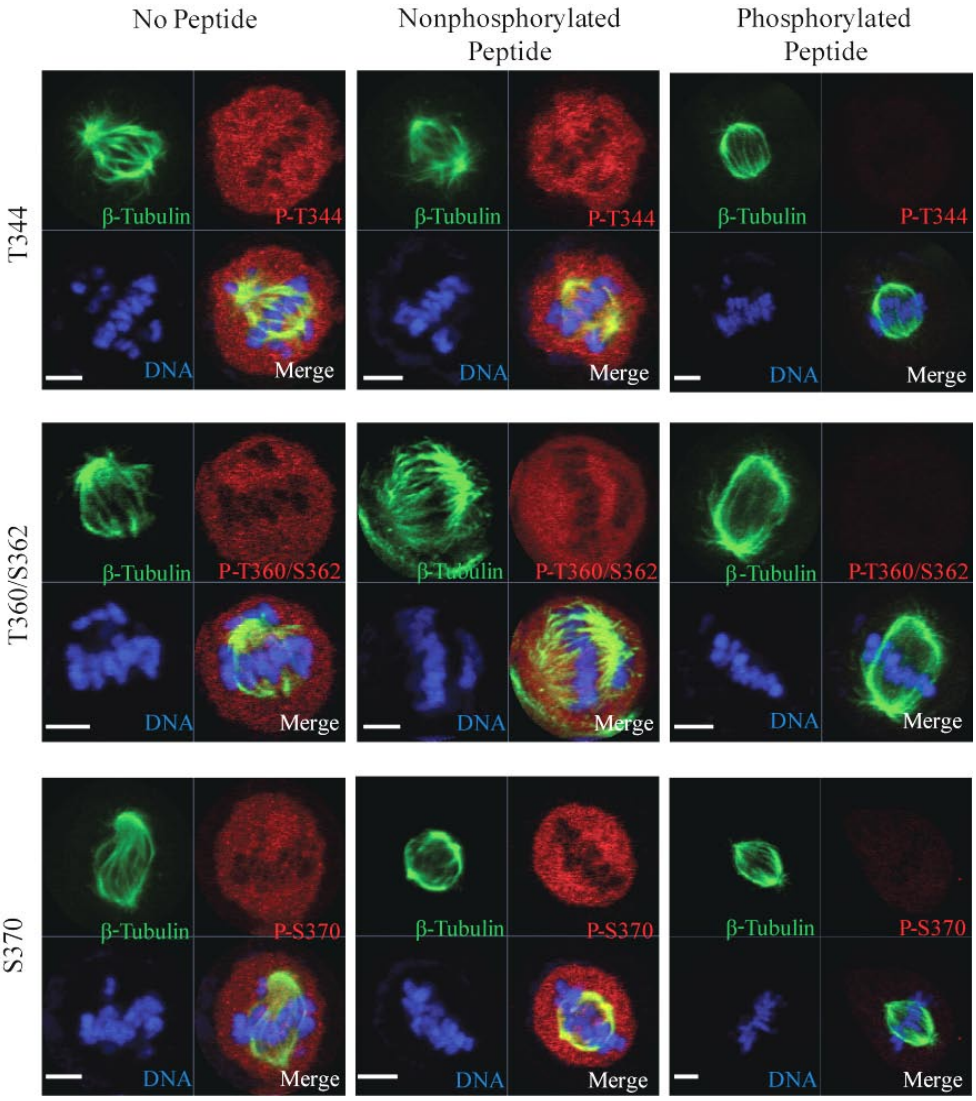


Figure 3.2 CK2α phosphospecific antibody peptide competition assay. CK2α phosphospecific antibodies were preincubated with a ten-fold molar excess of phosphorylated or non-phosphorylated versions of the peptide antigens used in antibody generation. Cells in mitosis were subjected to immunostaining with β-Tubulin and the pre-incubated phospho-CK2α antibodies, followed by confocal microscopy. Scale bars, 5 μm.

Figure 3.3

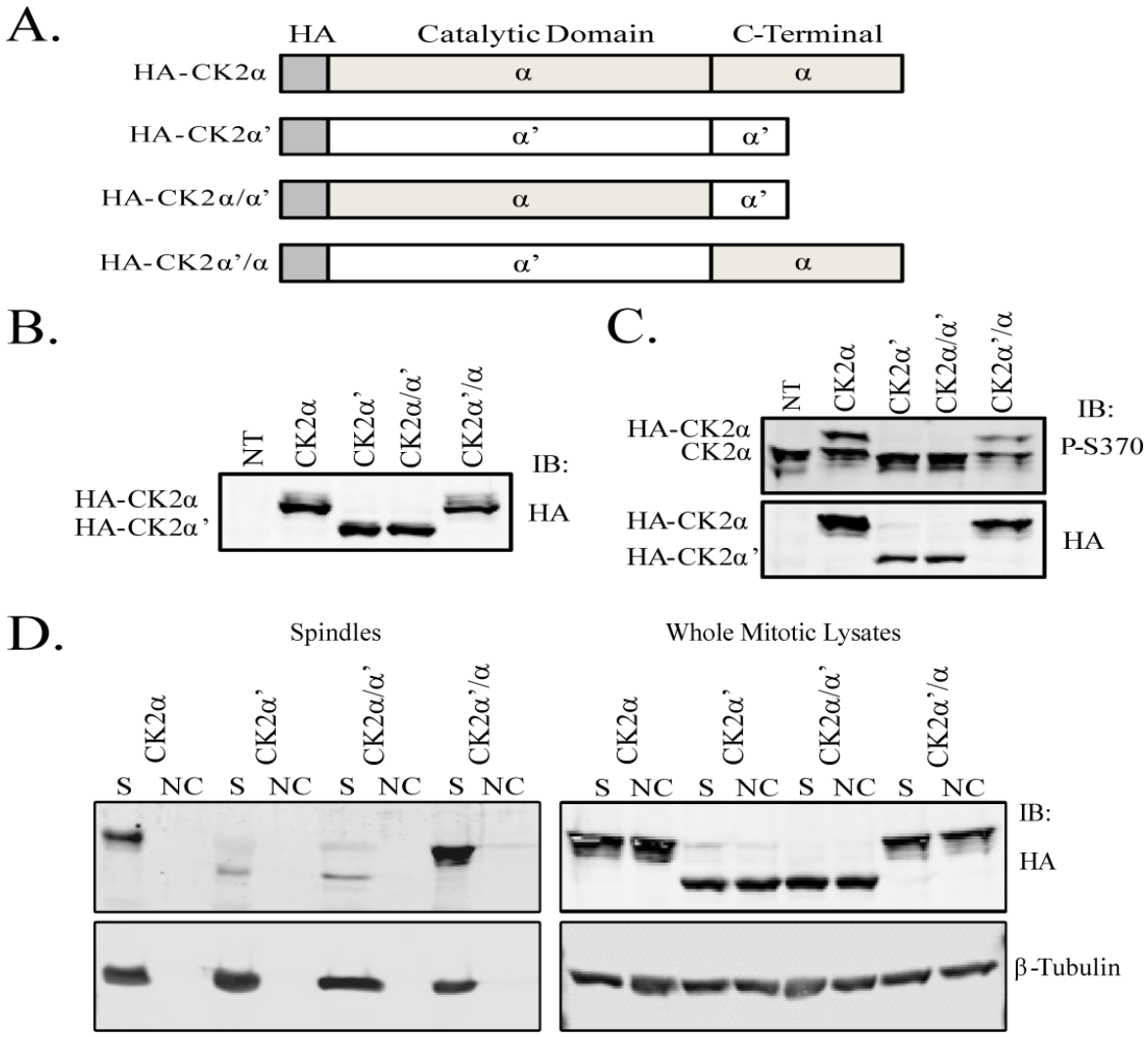


Figure 3.3 Mitotic spindle localization is unique to the CK2α catalytic subunit. (A) Schematic representation of HA-tagged CK2 catalytic subunits and subunit chimeras. (B) Lysates from cells expressing CK2 catalytic subunit variants were immunoblotted to detect the HA epitope. (C) Mitotic lysates from cells expressing CK2 catalytic subunit variants were immunoblotted with antibodies against phospho-CK2α and HA. (D) Purified mitotic spindles from cells transfected with CK2 catalytic subunits were immunoblotted for HA and β-Tubulin. S, spindles; NC, nocodazole control samples.

mitotic spindles at a level similar to CK2α. These results indicate that mitotic spindle localization of CK2 requires the unique C-terminus of CK2α.

To investigate if the CK2α C-terminus alone was sufficient for localization, the last 91 amino acids of CK2α (amino acids 300-391) were fused to DsRed (termed DsRed-C91). To investigate whether CK2α localization is phosphorylation-dependent, phosphorylation mutants of DsRed-C91 were generated; DsRed-C91-4D to mimic the phosphorylated state, and DsRed-C91-4A as a non-phosphorylatable form of DsRed-C91 (Figure 3.4A). The DsRed-C91 fusion protein was phosphorylated in mitotic cells (Figure 3.4B), and co-purified with isolated mitotic spindles, while DsRed alone did not (Figure 3.4C), indicating that the C-terminus of CK2α is capable of spindle localization in the absence of the remainder of the protein. Notably, this also indicates that CK2 kinase activity is not required for its localization to the mitotic spindle. Additionally, neither DsRed-C91-4D or DsRed-C91-4A showed any appreciable spindle co-purification. These results confirm that spindle localization of CK2α is dependent on its C-terminal phosphorylation state.

CK2α spindle localization requires the peptidyl-prolyl isomerase Pin1

Next, we investigated the molecular events responsible for phospho-CK2α localization. Pin1 is a peptidyl-prolyl isomerase that, due to its specificity for proline residues preceded by phosphoserine or phosphothreonine, has been extensively investigated as a mitotic regulatory enzyme (20, 40). Pin1 interacts with a variety of important mitotic signalling proteins (19), and it is thought that cis/trans conversion of adjacent prolyl bonds adds an additional level of regulation to phosphorylation sites (42).

Figure 3.4

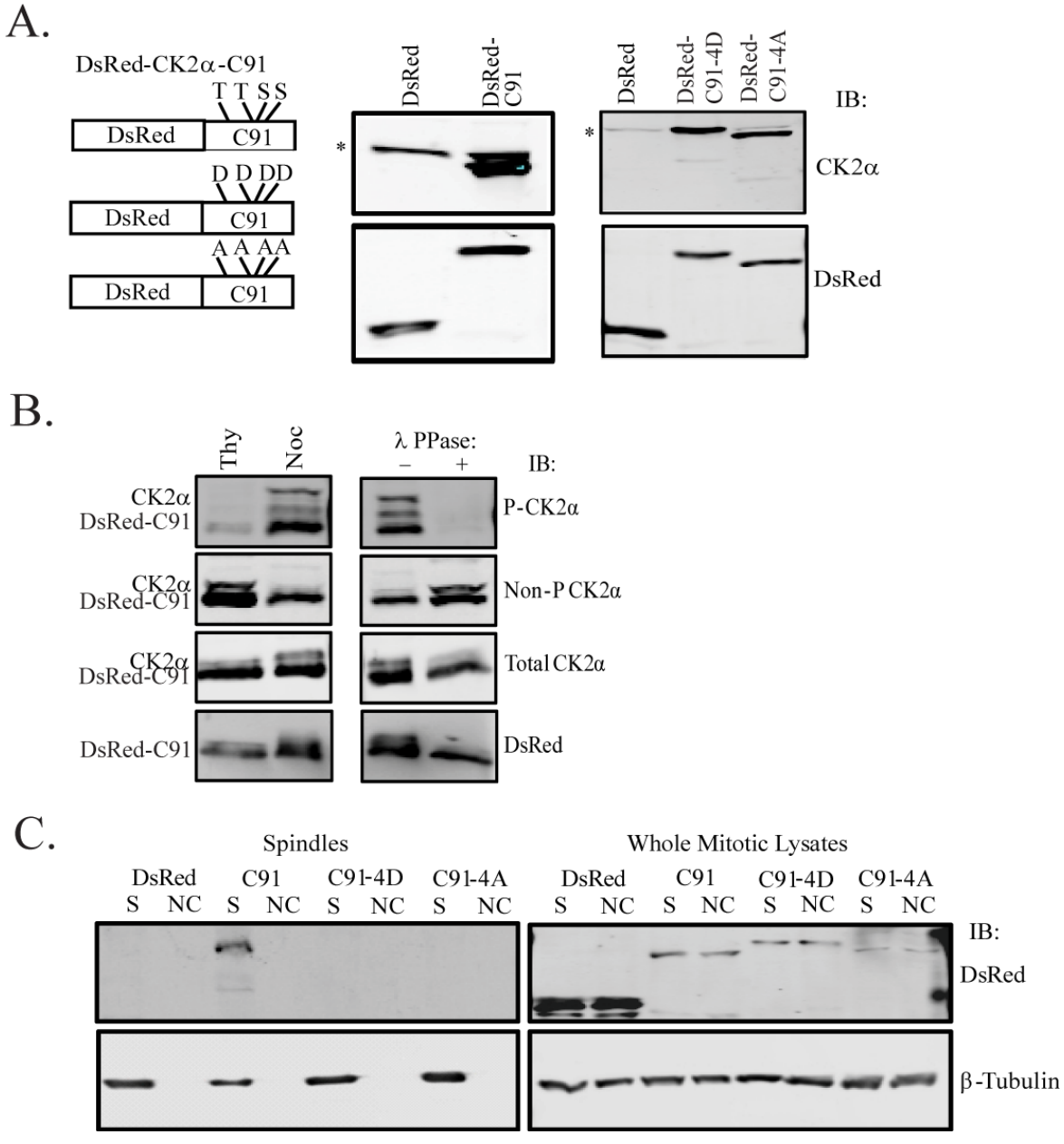

A.
DsRed-CK2α-C91
T T S S
DsRed
C91
D D DD
DsRed
C91
A A AA
DsRed
C91
DsRed
DsRed-C91
DsRed
DsRed-C91-4D
DsRed-C91-4A
IB:
CK2α
DsRed
B.
Thy
Noc
λ PPase:
–
+
IB:
CK2α DsRed-C91
P-CK2α
CK2α DsRed-C91
Non-P CK2α
CK2α DsRed-C91
Total CK2α
DsRed-C91
DsRed
C.
Spindles
Whole Mitotic Lysates
DsRed
C91
C91-4D
C91-4A
S NC S NC S NC S NC
DsRed
C91
C91-4D
C91-4A
S NC S NC S NC S NC
IB:
DsRed
β-Tubulin

Figure 3.4 The unique C-terminus of CK2α is responsible for mitotic spindle localization. (A) Schematic representation of DsRed-C91 and DsRed-C91 with the phosphorylation sites mutated to aspartic acid (4D) or alanine (4A). Lysates from transfected cells were immunoblotted for DsRed and CK2α. Band marked by * is endogenous CK2α. (B) Lysates from cells arrested in S phase with thymidine (Thy) or mitosis with nocodazole (Noc) were immunoblotted for phosphorylated CK2α, nonphosphorylated CK2α, total CK2α, and DsRed. Mitotic lysates were also dephosphorylated with λ Phosphatase before immunoblotting. (C) Purified mitotic spindles from cells expressing DsRed, DsRed-C91, DsRed-C91-4D or DsRed-C91-4A were immunoblotted with DsRed and β-Tubulin antibodies. S, spindles; NC, nocodazole control samples.

As Pin1 has previously been shown to interact with the CK2α C-terminus in a phosphospecific manner (25), we postulated that Pin1 may have a role in localization of phosphorylated CK2α. To investigate this, we employed stable cell lines expressing short hairpin RNA (shRNA) constructs against the 3' untranslated region of Pin1. A control cell line was also generated, expressing shRNA against Luciferase. The Pin1 shRNA cell lines displayed a marked decrease in the amount of Pin1 expressed in the cells (Figure 3.5A). Mitotic lysates from each cell line, immunoblotted for phospho-CK2α and harvested in the presence of phosphatase inhibitors, showed that CK2α phosphorylation was not prevented by knockdown of Pin1 (Figure 3.5B). Knockdown of Pin1 with either shRNA resulted in the loss of colocalization between phospho-CK2α and the mitotic spindle (Figure 3.5C). This loss of localization was confirmed with isolated spindles from each cell line, as phospho-CK2α did not co-purify with spindles from cells lacking Pin1 (Figure 3.5D). The spindle isolations also showed association of Pin1 with the mitotic spindle, and this was confirmed by immunostaining (Figure 3.5E). Cells expressing Pin1 shRNA were immunostained to control for antibody specificity. These results indicate that Pin1 localizes to the mitotic spindle, and is required for localization of phospho-CK2α to the mitotic spindle.

We next performed rescue experiments in which wild type or mutated versions of Pin1 were transfected into the Pin1 shRNA A cell line (Figure 3.6A). Pin1 is composed of two domains: a WW domain that binds to phosphorylated residues and a peptidyl-prolyl isomerase (PPIase) domain that isomerizes cis-trans conversion of prolyl bonds adjacent to phosphorylated serine or threonine (42). To determine if the WW domain is required, we used a Y23A mutant of Pin1, which disrupts Pin1 WW domain binding (21).

Figure 3.5

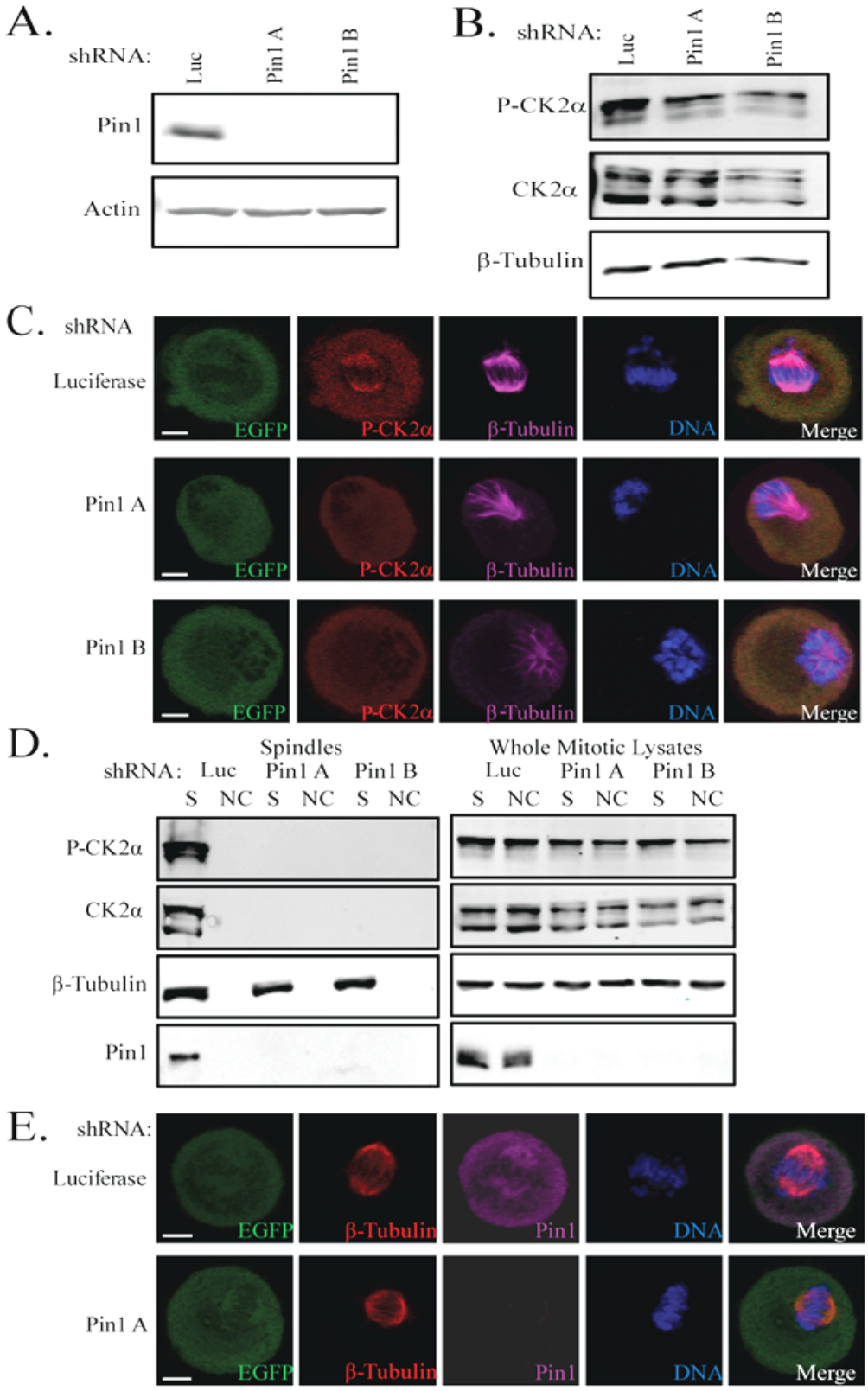
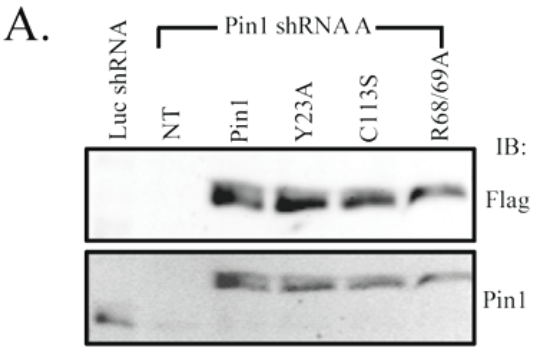
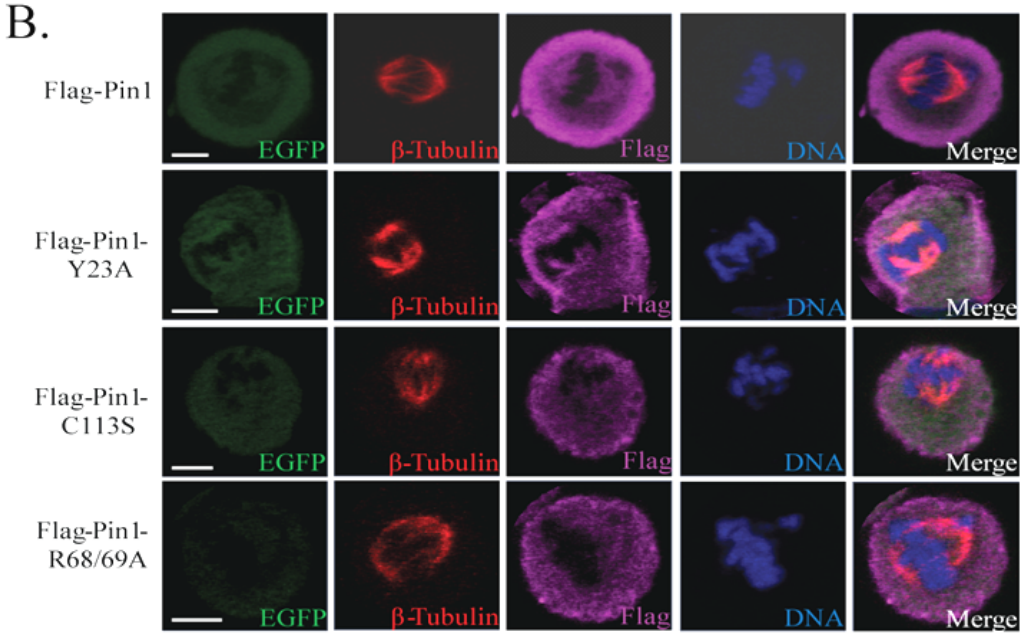

A.
shRNA:
Luc
Pin1 A
Pin1 B
Pin1
Actin
B.
shRNA:
Luc
Pin1 A
Pin1 B
P-CK2α
CK2α
β-Tubulin
C.
shRNA
Luciferase
EGFP
P-CK2α
β-Tubulin
DNA
Merge
Pin1 A
Pin1 B
D.
Spindles
Whole Mitotic Lysates
shRNA: Luc Pin1 A Pin1 B
S NC S NC S NC
P-CK2α
CK2α
β-Tubulin
Pin1
E.
shRNA:
Luciferase
EGFP
β-Tubulin
Pin1
DNA
Merge
Pin1 A

Figure 3.5 Pin1 recruits phosphorylated CK2α to the mitotic spindle. (A) Lysates from stable cell lines expressing short hairpin RNA (shRNA) plasmids targeting Luciferase (Luc), or the Pin1 3' untranslated region (Pin1 A, Pin1 B) were immunoblotted for Pin1 and β-Actin. (B) Luciferase and Pin1 knockdown cells were arrested in mitosis with nocodazole and lysates were immunoblotted for phosphorylated CK2α, total CK2α, and β-Tubulin. (C) Mitotic cells with stable expression of shRNA targeting Luciferase or Pin1 were immunostained for phosphorylated CK2α and β-Tubulin before confocal microscopy. The EGFP signal represents expression of the shRNA. (D) Purified mitotic spindles from cells with stable expression of shRNA against Luciferase or Pin1 were immunoblotted for phosphorylated CK2α, total CK2α, β-Tubulin and Pin1. S, spindles; NC, nocodazole control samples. (E) Mitotic Luciferase or Pin1-shRNA expressing cells were immunostained for Pin1 and β-Tubulin and subjected to confocal microscopy. Scale bars, 5 μm.

Figure 3.6



Flag Construct	Effect
Pin1	Wild type protein
Y23A	Disrupts binding via WW Domain
C113S	Disrupts isomerase activity of PPIase Domain
R68/69A	Disrupts binding via PPIase Domain



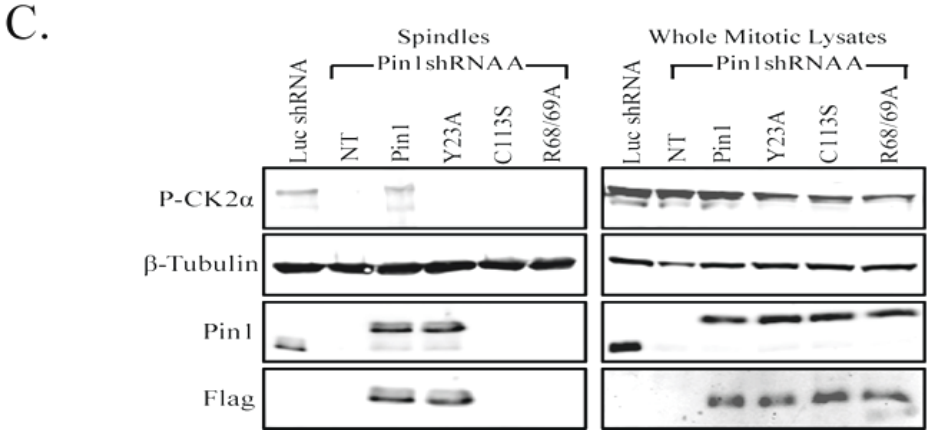


Figure 3.6 Proper localization of CK2 to the mitotic spindle requires Pin1 catalytic activity and WW domain binding. (A) Expression of Flag-Pin1 constructs in cells with stable knockdown of endogenous Pin1, and table of mutational effects on Pin1 function. Lysates were immunoblotted with antibodies against Flag and Pin1. Luc shRNA, lysate from control cells expressing a Luciferase shRNA; NT, not transfected. (B) Pin1 knockdown cells transfected with Flag-Pin1 variants were fixed in mitosis and immunostained for Flag and β-Tubulin. The EGFP signal represents expression of the shRNA. Scale bars, 5 μm. (C) Purified mitotic spindles from Luciferase knockdown cells, Pin1 knockdown cells, and Pin1 knockdown cells transfected with Flag-Pin1 variants were immunoblotted for phosphorylated CK2α, β-Tubulin, Pin1 and Flag.. S, spindles; NC, nocodazole control samples.

To investigate the effects of loss of PPIase activity, we used two different mutations in the Pin1 PPIase domain: C113S, (4) and R68/69A, (42), both of which are catalytically inactive towards phosphorylated substrates. We first assessed the ability of each Pin1 mutant to localize to the mitotic spindle. Like wild type Pin1, the Y23A mutant localized to the mitotic spindle, but both the C113S and R68/69A mutants were unable to similarly localize (Figure 3.6B). This suggests that spindle localization of Pin1 occurs independently of the WW domain, but requires PPIase activity. Following mitotic spindle isolation, the exogenous Pin1 mutants were then assessed for the ability to rescue the localization of phospho-CK2α to the spindle (Figure 3.6C). Expression of exogenous wild type Pin1 rescued the loss of spindle-associated phospho-CK2α observed with Pin1 knockdown, confirming that Pin1 is indeed required for spindle localization of phosphorylated CK2α. Neither PPIase mutant had the ability to localize phospho-CK2α to the mitotic spindle. Although Pin1-Y23A was found on the mitotic spindle at similar levels to wild type Pin1, it was also unable to localize phospho-CK2α to the spindle. Taken together, these results indicate that the presence of Pin1 is indeed required for localization of phospho-CK2α, and that proper localization requires both the WW and PPIase domains of Pin1.

Pin1 protects CK2 from dephosphorylation *in vivo*

We next sought to determine if Pin1 can isomerize the CK2α phosphorylation sites. While there is no direct assay to measure Pin1 activity in cells, other substrates of Pin1 have been identified through assessing dephosphorylation at these sites (36). As PP2A can only dephosphorylate residues in the trans configuration, residues isomerized to the cis configuration will be resistant to dephosphorylation (36). To disrupt the balance

between Cdk1 phosphorylation and PP2A dephosphorylation, cells expressing Pin1 shRNA were arrested in mitosis and then treated with 20 μM roscovitine, a Cdk1 inhibitor (24). Roscovitine treatment did not affect CK2α phosphorylation levels in cells expressing Luciferase shRNA, but cells expressing Pin1 shRNA showed a marked decrease in CK2α phosphorylation after roscovitine treatment, suggesting that the presence of Pin1 can protect CK2α from dephosphorylation (Figure 3.7A,B), However, this protection may be due to isomerization, or simply due to Pin1 physically blocking access of the phosphatase to the sites. To test this, we performed an *in vitro* assay with substoichiometric amounts of Pin1. Phospho-CK2α was immunoprecipitated from Pin1 shRNA expressing cells, and incubated with 1 nM, 10 nM, or 100 nM GST-Pin1 to allow isomerization to occur. GST and GST-Pin1-R68/69A were used as negative controls. Since the amount of CK2α in the assay greatly outnumbers the amount of Pin1 in this assay, Pin1 would not be able to protect CK2 simply from binding to the phosphorylation sites and blocking access of the phosphatase. However, if Pin1 can isomerize these sites, the low amounts of Pin1 in the assay should be able to protect CK2 from dephosphorylation. After addition of PP2A, Pin1 was unable to protect CK2α from dephosphorylation (Figure 3.7C)., indicating that protection from dephosphorylation is due to physical interaction, not isomerization. Regardless of the mechanism of action, what is clear is that interaction with Pin1 is crucial for proper localization of phosphorylated CK2α during mitosis.

Figure 3.7

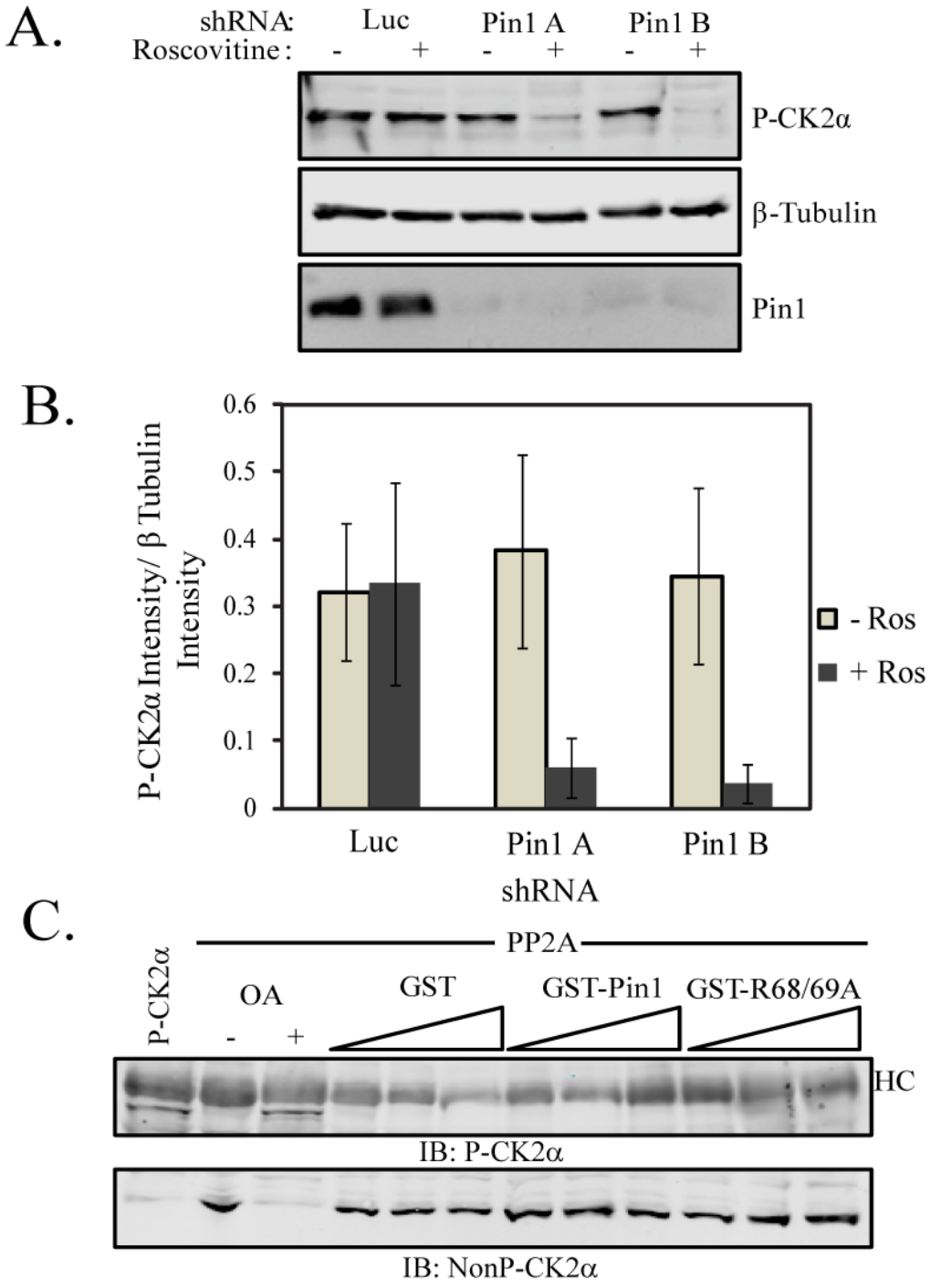

A.
shRNA:
Luc
Pin1 A
Pin1 B
Roscovitine:
- + - + - +
P-CK2α
β-Tubulin
Pin1
B.
P-CK2α Intensity/ β Tubulin Intensity
0.6
0.5
0.4
0.3
0.2
0.1
0
- Ros
+ Ros
Luc
Pin1 A
Pin1 B
shRNA
C.
PP2A
P-CK2α
OA
- +
GST
GST-Pin1
GST-R68/69A
HC
IB: P-CK2α
IB: NonP-CK2α

Figure 3.7 Pin1 protects CK2α from dephosphorylation *in vivo.* A. Lysates from mitotic luciferase and Pin1 knockdown cells treated with 20 μM roscovitine were immunoblotted for phosphorylated CK2α, β-Tubulin, and Pin1. B. Graph of relative phospho-CK2α intensity, normalized to β-Tubulin. Results are an average of three independent experiments. Error bars indicate one standard deviation from the mean. C. Phosphorylated endogenous CK2α, immunoprecipitated from mitotic cell lysates, was incubated with GST, GST-Pin1, or GST-Pin1-R68/69A before dephosphorylation with PP2A. Dephosphorylation was assessed by immunoblotting for phosphorylated and nonphosphorylated CK2α. OA, Okadaic Acid; HC, IgG heavy chain.

3.4 Discussion

In this study, we focused on the regulatory effect of four mitotic phosphorylation sites on the unique C-terminus of the CK2 catalytic isoform CK2α. While previous studies have observed CK2 localized at the mitotic spindle (10, 43), the mechanism for localization was not determined. In this report, we show for the first time that phosphorylation of CK2α can regulate its subcellular localization. This research also highlights a rare example of functional divergence between the two catalytic isoforms of CK2. While CK2α and CK2α' share remarkable sequence and structural similarity throughout most of the proteins (18), they have completely divergent C-termini, meaning that CK2α' does not contain the four mitotic phosphorylation sites in CK2α. Previous work on CK2 localization has produced mixed results as to whether CK2α alone, or both catalytic subunits can localize to the mitotic spindle (10, 43). Here we show that CK2 localization to the mitotic spindle is mediated through CK2α. Since both the CK2α C-terminus and the four phosphorylation sites it contains are required for localization, CK2α' is incapable of mediating its own spindle localization. Our results did show a small amount of CK2α' in isolated spindle preparations, probably due to the formation of mixed tetramers between CK2α' and phosphorylated CK2α. This may also explain the disparity between previous reports. It is intriguing that in CK2α' knockout mice, the presence of CK2α largely compensates for the loss (41), while CK2α' cannot compensate for loss of CK2α, which is embryonic lethal (16). This suggests that the specialized functions of the CK2α C-terminus may be the cause of this lethality. Additionally, the importance of these sites is highlighted by their sequence conservation in birds and

mammals (18, 23). Clearly, the C-terminus of CK2α has an important mitotic role in the cells of higher order animals.

Pin1 is a peptidyl-prolyl isomerase that, due to its specificity for proline residues preceded by phosphoserine or phosphothreonine, has been extensively investigated as a mitotic regulatory enzyme (20, 40). Pin1 interacts with and isomerizes a variety of important mitotic signalling proteins (19), and it is postulated that cis/trans conversion of prolyl bonds adjacent to phosphorylated residues adds an additional level of regulation to these phosphorylation sites (42). As Pin1 binds specifically to phosphorylated CK2α, we investigated whether loss of Pin1 would affect CK2α spindle localization. Indeed, upon knockdown of Pin1, phosphorylated CK2α loses the ability to localize to the mitotic spindle. Furthermore, we observed colocalization of Pin1 itself to the mitotic spindle. The requirement of Pin1 for phospho-CK2α localization to the mitotic spindle explains why the phosphomimetic version of the CK2α C-terminus did not localize to the mitotic spindle. While phosphomimetic mutations can effectively mimic phosphorylation sites for some interacting proteins, Pin1 cannot bind to phosphomimetic CK2α (25). Pin1 requires an acidic residue for binding, and shows much higher affinity for phosphoserine or phosphothreonine than aspartic acid (42). Previous work has demonstrated that the interaction between the C-terminus of CK2α and Pin1 requires the WW domain (25). The Y23A mutation, which disrupts the binding ability of the Pin1 WW domain, localized to the mitotic spindle yet failed to recruit CK2α. Interestingly, localization of Pin1 itself to the mitotic spindle seems to require mainly the PPIase domain, as neither PPIase domain mutant was able to rescue Pin1 spindle localization. It appears that both Pin1 isomerase activity and WW domain binding are required for Pin1-phosphorylated CK2α complexes

to localize to the mitotic spindle – isomerase activity is required to localize Pin1, while WW domain binding is required for CK2α to be brought along. Pin1 has many interactors/substrates on the mitotic spindle, and the overlap with known CK2 interactors/substrates is striking, including Plk1 (17, 31), Wee1 (31, 39), and Cdc25C (30, 36). It is intriguing to imagine that Pin1 may target phosphorylated CK2α to the spindle by binding to its spindle substrates, and that CK2 may catalyze additional phosphorylation events along these Pin1 substrates. Identification of these substrates and investigation of this hypothesis is an area of great interest in our future work. What is clear, however, is that Pin1 localizes CK2α to the mitotic spindle in a phosphorylation-dependent manner, and in doing so may focus the activity of a phenomenally pleiotropic kinase towards important mitotic targets. Not only does this work contribute to the growing evidence for important mitotic roles for both CK2 and Pin1, but it is the first evidence for regulation of CK2 localization by phosphorylation, and the first report of Pin1 acting to localize a phosphorylated protein to the mitotic spindle. It is certainly a possibility that Pin1 may have a role in localization of other important mitotic regulatory proteins. Further investigation of this possibility may offer new insight on the regulation of mitotic cell division.

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Chapter 4: Hierarchical phosphorylation between Cdk1 and CK2 may contribute to the regulation of mitosis

4.1 Introduction - Protein phosphorylation is catalyzed by protein kinases, which are exquisitely regulated in the cell to ensure proper phosphorylation in response to a given stimulus. Kinase activity can be controlled by activating and/or inhibitory phosphorylation events on the kinase itself, through protein-protein interactions with regulatory subunits and other proteins, and through changes in subcellular localization. Several well-studied kinases respond to chemical second messengers such as cyclic AMP (cAMP). Additional modes of regulation include modulation of protein expression at the transcript level, and turnover of the protein product by targeted degradation. These mechanisms ensure that each kinase is active and able to phosphorylate its substrates only at the correct time and location. While all mammalian protein kinases phosphorylate proteins at tyrosine and/or serine/threonine residues, different kinase families have evolved to require specific sequence determinants in order to target a particular kinase to its substrates (31). For example, the large family of Cyclin-dependent kinases (Cdks), which are vital for cell cycle progression, phosphorylate substrates at serine/threonine residues that are immediately followed by a proline residue. To distinguish between these sites, Cdks have each evolved additional individual sequence determinants. For example, the optimal consensus sequence for phosphorylation by Cdk1, the master regulatory kinase controlling the process of mitosis, is S/T-P-V-K/R (31). Through identification of the optimal consensus sequence, it is possible to predict possible substrates for a particular kinase.

Several decades of work have resulted in a huge body of knowledge regarding kinase-substrate reactions and their individual functional effects on the cell, and large

scale phosphoproteomic screens have contributed large datasets of potential phosphorylation sites. It is estimated that one third of intracellular proteins are phosphorylated, many on several distinct sites (19). While it is apparent that the actions of protein kinases are intricately involved in every fundamental cellular process (17), our understanding of the interplay between distinct protein kinases has lagged considerably. Hierarchical protein phosphorylation is a phenomenon in which a kinase phosphorylates a substrate based on its unique sequence determinants, and the addition of phosphate creates adequate sequence determinants for nearby phosphorylation events to occur. These events can involve two or more distinct kinases, or can result in processive phosphorylation events catalyzed by only one kinase. A few protein kinases, namely GSK3 and CK1, catalyze primed phosphorylation events almost exclusively, as phosphorylation by these kinases usually requires prior phosphorylation of a nearby residue (12, 13). A few other kinases, including protein kinase CK2, can also utilize phosphorylated determinants for phosphorylation.

CK2 is a ubiquitously expressed serine/threonine kinase that has a multitude of cellular substrates, and due to this, it participates in a variety of cellular processes, including proliferation, apoptosis, transcription, and translation (44). Unlike many protein kinases, CK2 is not regulated by either second messengers or activating or inhibitory phosphorylation events, and is largely regarded as constitutively active. However, some CK2 substrates show evidence of regulated phosphorylation (14, 20). Additionally, the requirement for CK2 activity in a number of highly regulated cellular processes, in particular the regulation of cell division, indicates that CK2 must be regulated in some way, at least for certain substrates. Indeed, previous work in our laboratory has

demonstrated that during mitosis, CK2 is regulated through phosphorylation (2, 24), protein-protein interactions (30), and subcellular localization (see Chapter 3).

CK2 is an acidophilic kinase, requiring one or more acidic residues on the C-terminal side of the phosphoacceptor site in order to recognize and phosphorylate its substrates. Accordingly, the minimal consensus sequence for CK2 phosphorylation is S/T-X-X-Acidic. X can be any amino acid, however proline, lysine, or arginine at the +1 position are unfavourable (26, 28). The acidic determinant can also be in the +1 position instead of the +3 position, but is only rarely found at the +2 position. The acidic determinants seem to have an additive effect, and due to this, many known CK2 sites consist of a serine/threonine residue followed by a string of acidic residues (29). Interestingly, phosphoserine and phosphotyrosine (but not phosphothreonine) can substitute for the acidic determinant at the +3 position, enabling CK2 to participate in hierarchal signalling events (23, 27). These events have not been systematically studied, but anecdotal evidence suggests that hierarchal phosphorylation involving CK2 can contribute to the regulation of cellular processes. For example, in an elegant study of the mitotic regulatory kinase Wee1A, Watanabe *et al.* demonstrated that phosphorylation of Wee1A by Cdk1 at serine 123 primes subsequent phosphorylation by CK2 at serine 121 (48). Wee1A regulates Cdk1 activity through inhibitory phosphorylation, controlling the onset of mitosis (35). Phosphorylation by Cdk1 and CK2, as well as upstream phosphorylation by Plk1, promotes degradation of Wee1A, resulting in the onset of mitosis (48). The requirement for Cdk1 phosphorylation for subsequent CK2 phosphorylation puts CK2, a constitutively active kinase, under the same regulatory controls as Cdk1, which is exquisitely controlled through a variety of mechanisms.

We hypothesize that hierarchical phosphorylation between Cdk1 and CK2 may modulate CK2 substrate recognition during mitosis, allowing phosphorylation of mitotic substrates. To examine the role of hierarchical phosphorylation between Cdk1 and CK2 in the regulation of mitosis, we have systematically determined optimal consensus sequences for both primed phosphorylation by CK2 in general, and for Cdk/CK2 hierarchical phosphorylation. Using the consensus sequence for Cdk/CK2 hierarchical phosphorylation, we then probed the human genome for potential substrates. The results indicate that hierarchical phosphorylation between Cdk1 and CK2 could contribute to the phosphorylation of several mitotic proteins, and therefore may contribute substantially to the regulation of mitosis.

4.2 Materials and Methods

Purification of Active CK2 Holoenzyme – CK2 holoenzyme, consisting of GST-CK2α and His-CK2β, was purified from bacterial culture as described in Turowec *et al.* (46). Briefly, BL21 *E. coli* cells containing the plasmid pDB1 (encoding GST-CK2α) were grown at 37°C in 2XYT media containing 0.1 mg/mL ampicillin to an OD_{600} of 0.8, at which point 0.5 mM IPTG was added to induce protein expression for 3 hours at 37°C. BL21 cells containing the plasmid pAB46 (encoding His-CK2β) were grown at 37°C in LB media containing 50 μg/mL kanamycin to an OD_{600} of 0.8, then induced by addition of 0.5 mM IPTG for 24 hours at 15°C. Bacterial cultures were then collected by centrifugation, resuspended in lysis buffer (PBS with 30 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM PMSF), and combined. The bacteria were lysed by two passes through a French press, tumbled for 15 minute at 4°C in PBS supplemented with 1% Triton X-100, and finally cleared by centrifugation. The supernatant was then incubated with glutathione-agarose beads (Sigma) for 1 hour at 4°C with constant rotation. The beads were washed with 20 column volumes of lysis buffer, and the enzyme was eluted from the beads in 1 mL fractions with first 10 mM glutathione and then 30 mM glutathione (both in 50 mM Tris-HCl pH 8.0 with 1 mM DTT). Fractions containing CK2, identified by SDS-PAGE and Coomassie blue staining, were dialyzed overnight into CK2 storage buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% Glycerol) and stored at -20°C. Enzyme concentration was determined by absorbance at 595 nm, measured on a Victor3 V 1420 multilabel counter (Perkin Elmer) using BSA standards. To maintain linear activity, the enzyme was diluted 1:5000 in CK2

Dilution Buffer (5 mM MOPS pH 7.0, 200 mM NaCl, 1 mg/mL BSA) immediately before use in kinase assays.

Biotinylated peptide kinase assays – Biotinylated peptides were synthesized using standard Fmoc (9-fluorenylmethyloxycarbonyl) chemistry at a 2 μmol scale on an Intavis Multipep Synthesizer. The sequences of all peptides used in this study can be found in Table 4.1. Biotin was added to the N-terminus of each peptide. Peptides were cleaved from the resin using trifluoroacetic acid, and peptide identities were confirmed by mass spectrometry. Peptides were resuspended in DMSO and concentrations were determined by absorbance at 280 nm, using an N-terminal tryptophan residue. Peptide concentration was adjusted to 50 mM, and aqueous 2.5 mM stock solutions were made in 20 mM HEPES pH 7.4. Kinase assays were performed for 10 minutes at 30°C in a final reaction volume of 10 μl containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, and 1 μCi [γ^{32}P]-ATP (specific activity 3000 Ci/mol, Perkin-Elmer). Substrate peptides were used at 0.5 mM. Reactions were initiated by the addition of CK2, and stopped by the addition of 2 μL of 0.5 M EDTA (pH 8.0). 4 μL aliquots of each reaction were spotted onto a SAM2 streptavidin membrane (Promega). The membrane was washed twice in 0.1% SDS in TBS, twice in 2M NaCl, twice in 2M NaCl and 1% H_3PO_4, and twice in distilled water, and then dried under a heat lamp. Following 24 hours of exposure to a phosphor storage screen, CK2 phosphorylation was visualized using a Storm Phosphorimager (Molecular Dynamics) and [γ^{32}P] incorporation for each peptide was determined using ImageQuant TL software (Amersham Biosciences). All phosphorylation assays were performed in triplicate. For kinetic studies, each peptide was assayed for phosphorylation at a minimum of six different concentrations, and five

replicates of each concentration were used in the analysis. Km and Vmax values for each peptide were determined by nonlinear regression analysis fit to a Michaelis-Menten model for enzyme kinetics using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Each data set was subjected to a replicates test for lack of fit, and the Michaelis-Menten model was deemed adequate for all peptides studied.

Bioinformatics - A peptide match program was designed to search the SwissProt and NCBI databases for human proteins containing potential Cdk/CK2 hierarchical phosphorylation events. The program was engineered to search for peptides matching the sequence S-X-X-X-X-S-P, and score the results at positions 2-5 based on the presence of CK2 consensus determinants. Following a proteome-wide search for matching peptides, the program returned the GI number, SwissProt protein ID, peptide sequence, position within the protein, and CK2 site score for each hit. Results were then sorted by their CK2 score, and results with no or negative CK2 determinants were excluded from the study. The resultant peptide sequences were sorted by Gene Ontology using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (8, 16).

TABLE 4.1 Sequences of peptides used in this study.

Peptide Sequence[a]	Purpose in Study
WDDDSDDDDDAAA	Canonical Positive D
WDDDADDDDDAAA	Canonical Negative D
WEEESEEEEEAAA	Canonical Positive E
WEEEAEEEEEAAA	Canonical Negative E
WDDDSpSpSpSpSpSAAA	Primed Positive
WDDDApSpSpSpSpSAAA	Primed Negative
WDDDSSSSSSAAA	Primed Negative
WDDDSApTpTpTpTAAA	STY Comparison
WDDDSApYpYpYpYAAA	STY Comparison
WDDDSAAAAAAAA	Primed Negative
WDDDSApSpSpSpSAAA	1[b]/ STY Comparison
WDDDSpSApSpSpSAAA	2
WDDDSpSpSApSpSAAA	3
WDDDSpSpSpSApSAAA	4, Kinetic measurements
WDDDSpSpSpSpSAAAA	5
WDDDSAApSpSpSAAA	12
WDDDSApSApSpSAAA	13
WDDDSApSpSApSAAA	14
WDDDSApSpSpSAAAA	15
WDDDSpSAApSpSAAA	23, Kinetic measurements
WDDDSpSApSApSAAA	24
WDDDSpSApSpSAAAA	25, Kinetic measurements
WDDDSpSpSAApSAAA	34
WDDDSpSpSApSAAAA	35
WDDDSpSpSpSAAAAA	45, A/P Comparison
WDDDSAAApSpSAAA	123
WDDDSAApSApSAAA	124
WDDDSAApSpSAAAA	125
WDDDSApSAApSAAA	134
WDDDSApSApSAAAA	135
WDDDSApSpSAAAAA	145
WDDDSpSAAApSAAA	234
WDDDSpSAApSAAAA	235
WDDDSpSApSAAAAA	245, Kinetic measurements, A/P Comparison
WDDDSpSpSAAAAAA	345
WDDDSpSAAAAAAA	2345
WDDDSApSAAAAAA	1345

Peptide Sequence[a]	Purpose in Study
WDDDSAApSAAAAA	1245, Primed Positive, Kinetic measurements, A/P comparison
WDDDSAAApSAAAA	1235
WDDDSAAAApSAAA	1234
WDDDSAASAAAAA	Primed Negative
WDDDSpSpSpSPVKAA	A/P Comparison
WDDDSAApSPVKAA	A/P Comparison
WDDDTApSpSpSpSAAA	S/T Comparison
WFVPSDApSPPKTK	TopoII #1 Experimental
WFVPSDASPPKTK	TopoII #1 S control
WFVPSDADPPKTK	TopoII #1 D control
WFVPADApSPPKTK	TopoII #1 A control
WPPKTKTpSPKLAN	TopoII #2 Experimental
WPPKTKTSPKLAN	TopoII #2 S control
WPPKTKTDPKLAN	TopoII #2 D control
WPPKAKTpSPKLAN	TopoII #2 A control
WDEKTDDEDFVPA	TopoII Canonical Experimental
WDEKADDEDFVPA	TopoII Canonical A control
WGFGSSpSPVKAPA	Wee1 Experimental
WGFGSSSPVKAPA	Wee1 S control
WGFGSSDPVKAPA	Wee1 D control
WGFGASpSPVKAPA	Wee1 A control
WAPSTNSpSPVLKT	Separase Experimental
WAPSTNSSPVLKT	Separase S control
WAPSTNSDPVLKT	Separase D control
WAPAANApSPVLKT	Separase A control
WLSPTISpSPPNALP	Cdc27 Experimental
WLSPTISSPPNALP	Cdc27 S control
WLSPTISDPPNALP	Cdc27 D control
WLSPAISpSPPNALP	Cdc27 A control
WDSPSQGpSPALYRN	Repo-Man (CDCA2) Experimental
WDSPSQGSPALYRN	Repo-Man S control
WDSPSQGDPALYRN	Repo-Man D control
WDSPAQGpSPALYRN	Repo-Man A control
WDDDSDApSPVKAA	P +1 Acidic
WDDDSDASPVKAA	P +1 Acidic S Control
WDDDSRApSPVKAA	P +1 Basic
WDDDSFApSPVKAA	P +1 Bulky Hydrophobic
WDDDSMApSPVKAA	P +1 Methionine

Peptide Sequence[a]	Purpose in Study
WDDDSQApSPVKAA	P +1 Uncharged Polar
WDDDSHApSPVKAA	P +1 Charged Polar
WDDDSpSApSPVKAA	P +1 Phosphate, A/P Comparison
WDDDSPApSPVKAA	P +1 Proline
WDDDSADpSPVKAA	P +2 Acidic
WDDDSADSPVKAA	P +2 Acidic S Control
WDDDSARpSPVKAA	P +2 Basic
WDDDSAFpSPVKAA	P +2 Bulky Hydrophobic
WDDDSAMpSPVKAA	P +2 Methionine
WDDDSAQpSPVKAA	P +2 Uncharged Polar
WDDDSAHpSPVKAA	P +2 Charged Polar
WDDDSApSpSPVKAA	P +2 Phosphate
WDDDSAPpSPVKAA	P +2 Proline
WDDDSpSAAApSPVK	P at +6, +1 pS
WDDDSEAAApSPVK	P at +6, +1 E
WDDDSEAAASPVK	P at +6, +5 S Control
WDDDSpSAApSPVKA	P at +5, +1 pS
WDDDSEAApSPVKA	P at +5, +1 E
WDDDSEAASPVKA	P at +5, +4 S Control
WDDDSpSApSPVKAA	P at +4, +1 pS
WDDDSEApSPVKAA	P at +4, +1 E
WDDDSEASPVKAA	P at +4, +3 S Control
WDDDSAApSApSPVK	P at +6, +3 pS
WDDDSAAEApSPVK	P at +6, +3 E
WDDDSAAEASPVK	P at +6, +5 S Control
WDDDSAApSpSPVKA	P at +5, +3 pS
WDDDSAAEpSPVKA	P at +5, +3 E
WDDDSAAESPVKA	P at +5, +4 S Control

[a]All peptides contain an N-terminal biotin. The phosphoacceptor site is indicated in red.
[b]Numbered peptides are modelled on the peptide WDDDSpSpSpSpSpSAAA. Numbers indicate the positions at which phosphoserine has been replaced with alanine.

4.3 Results

Potential substrates for Cdk1/CK2 hierarchical phosphorylation

Protein kinase CK2 is an acidophilic kinase that preferentially phosphorylates substrates at serine or threonine residues with aspartic acid and glutamic acid residues C-terminal to the phosphoacceptor site. For substrates that conform to the canonical acidic consensus, phosphorylation is particularly favoured if an acidic determinant is present at the +3 position (S/T-X-X-D/E), but the positioning can vary (the +1 position is also highly favoured), and in many cases CK2 substrate sequences feature multiple acidic residues C-terminal to the phosphorylation site (29). All of these factors contribute to a large amount of flexibility in what constitutes a functional site for CK2 phosphorylation. Interestingly, CK2 may also use phosphoserine as an acidic determinant (23, 48), but the consensus requirements for these phosphorylation events have never been systematically determined. In this study, we chose to examine whether hierarchical phosphorylation between Cdk1 and CK2 contributes to the mitotic regulation of CK2, allowing it to specifically phosphorylate mitotic substrates. To begin, we first searched the PhosphoSite (15) database of known *in vivo* phosphorylation sites for mitotic proteins containing potential sites for Cdk1/CK2 hierarchical phosphorylation. While primed phosphorylation by CK2 was shown with a peptide containing phosphoserine at the +3 position (23), in the only known example of this phenomenon, the mitotic kinase Wee1A is phosphorylated by Cdk1 at the +2 position compared to the CK2 phosphoacceptor site (pS-S-pS-P-V-K) (48). As mentioned above, this spacing is unusual for CK2, which does not typically favour phosphodeterminants at the +2 position. For these reasons, we elected to search for proteins containing the sequence S/T-X-X-S-P. We then sorted the

results to include only proteins with previously reported phosphorylation events at either site, specifically during mitosis. A list of selected proteins previously shown to be phosphorylated during mitosis at sites matching the pattern S/T-X-X-S-P is shown in Table 4.2. The complete list is included in the supplemental material in Appendix A. As evident from Table 4.2, several important mitotic proteins are potentially regulated in this manner. To validate these results, biotinylated peptides of target sequences from selected candidates were incubated with CK2 and [γ^{32}P]-ATP. For each peptide, we included two negative controls; one with an unphosphorylated Cdk1 site, and one with the CK2 site mutated to alanine as a negative control. To examine whether a normal acidic determinant can effectively replace the Cdk1-phosphorylated residue, we included a peptide with the Cdk1 site mutated to aspartic acid. Topoisomerase IIα, a known mitotic substrate of CK2, was chosen for further study, as PhosphoSite indicated that two sites, immediately downstream from a known mitotic CK2 site at T1343 (7), both fit the potential consensus sequence (Figure 4.1A). Furthermore, in both these sites the proline-directed serine can be phosphorylated by Cdk1 (49). However, upon incubation with CK2, the only Topoisomerase IIα peptide phosphorylated was the peptide containing the known canonical site at T1343 (sequence: T-D-D-E-D) (Figure 4.1B). Additionally, peptides with promising sequences from the mitotic regulatory proteins Separase, Repo-Man, and Cdc27 proved to be poor substrates for CK2 primed phosphorylation, with only Repo-Man displaying any detectable phosphorylation (Figure 4.1C, D, E). Interestingly, even the known Wee1A phosphorylation site was not phosphorylated in phosphopeptide form (Figure 4.1F). Collectively, these results suggest that Cdk1/CK2 hierarchical

TABLE 4.2 Potential mitotic substrates for Cdk1/CK2 hierarchical phosphorylation[a].

Protein	Sequence	Function	Reference
CDCA2 (Repo-Man)	129-pSQGpSPAL	Mitosis	(9)
Cdc27	366-pTISpSPPN	Anaphase promoting complex component	(9, 45)
CDK7	161-pSFGpSPNR	S/T Kinase	(6, 9)
CENPC1	535-pSEEpSPVY	Kinetochore assembly	(33)
CENPE	2651-pSLPpSPHP	Microtubule motor protein	(33)
CENPF	2993-pSRGpSPLL	Kinetochore assembly	(9, 33)
CEP350	1253-pTPpTpSPLpSP	Microtubule-centrosome attachment	(9)
CHED (Cdc2L5)	436-pSpSIpSPpSpT	S/T Kinase	(6, 9)
CLASP1	1088-pSVGpSPpS	Microtubule/Kinetochore binding protein	(9)
DSN1	77-pSpSLpSPVE	Kinetochore assembly	(6)
Dynamin 2	761-pSpSHpSPpTP	Microtubule binding protein	(9)
GAS2L1	489-pSpSPpSPEL	Cell cycle arrest	(9)
GAS2L3	567-SpSVpSPVK	Cell cycle arrest	(9)
Kinesin 13B	1379-pSIpSpSPNV	Microtubule binding, kinesin-like	(9)
KIF18A	681-pTLKpSPPS	Kinesin	(9)
KIF20A	864-pTDCpSPpYA	Microtubule binding protein	(9)
MAP1B	1912-pTpTKpSPpSD	Microtubule binding protein	(9)
MAP4	822-pSpSRpSPpSpT	Microtubule binding protein	(9)
NEK1	661-pTGGpSPSK	S/T Kinase	(6)
	834-pSEIpSPEG		(6)
Sgo1 (Shugoshin)	433-pTQQpSPHL	Chromosome cohesion in mitosis	(9)
TOP2A	1351-pSDApSPPK	DNA Topoisomerase	(9, 33)
	1358-pTKTpSPKL		(9)
Wee1	136-pSpSFpSPVR	S/T Kinase	(6)

[a] Selected results from PhosphoSite (15), www.phosphosite.org, Search: S/T-X-X-S-P.

Figure 4.1

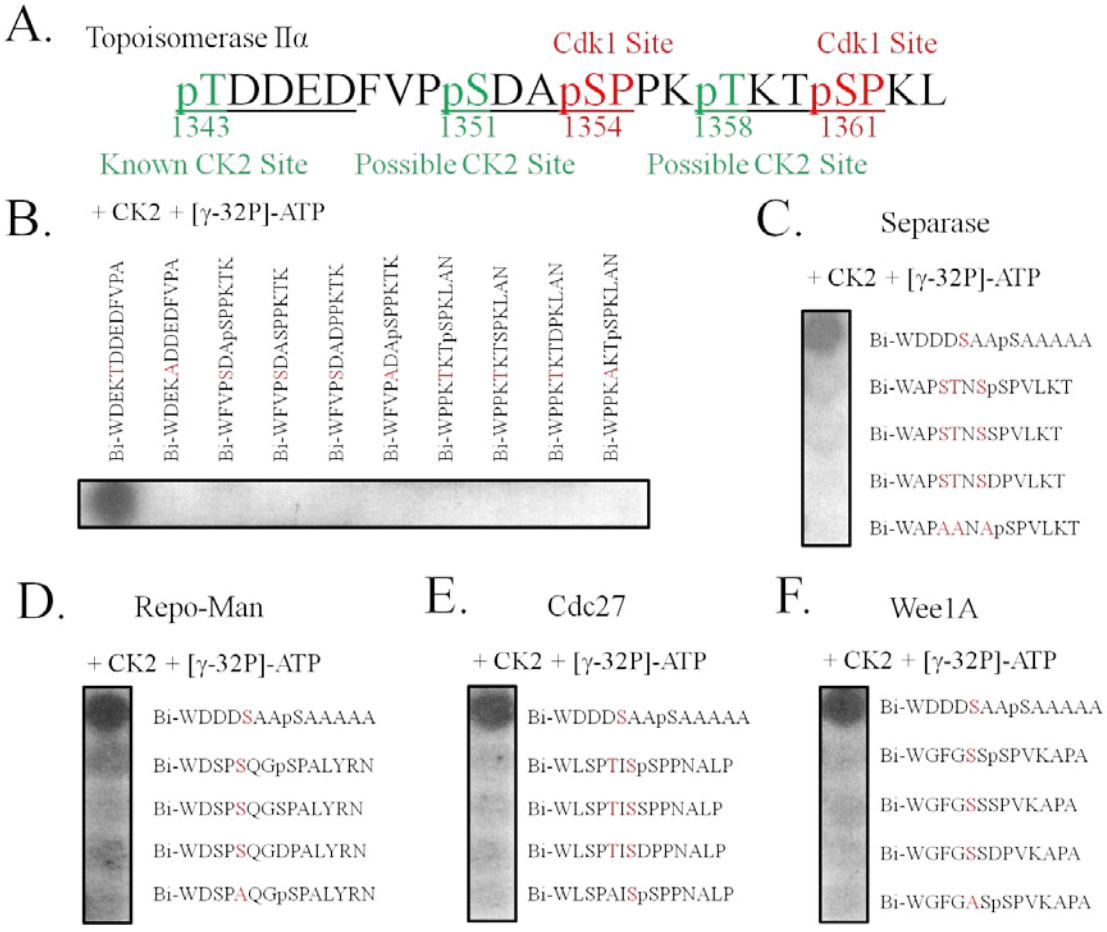
A.
Topoisomerase IIα
Cdk1 Site
Cdk1 Site
pTDDEDFVPpSDApSPPKpTKTpSPKL
1343
1351
1354
1358
1361
Known CK2 Site
Possible CK2 Site
Possible CK2 Site
B.
+ CK2 + [γ-32P]-ATP
Bi-WDEKTDDEDFVPA
Bi-WDEKADDEDFVPA
Bi-WFVPSDApSPPKTK
Bi-WFVPSDASPPKTK
Bi-WFVPSDADPPKTK
Bi-WFVPADApSPPKTK
Bi-WPPKTKTpSPKLAN
Bi-WPPKTKTSPKLAN
Bi-WPPKTKTDPKLAN
Bi-WPPKAKTpSPKLAN
C.
Separase
+ CK2 + [γ-32P]-ATP
Bi-WDDDSAApSAAAAA
Bi-WAPSTNSpSPVLKT
Bi-WAPSTNSSPVLKT
Bi-WAPSTNSDPVLKT
Bi-WAPAANApSPVLKT
D.
Repo-Man
+ CK2 + [γ-32P]-ATP
Bi-WDDDSAApSAAAAA
Bi-WDSPSQGpSPALYRN
Bi-WDSPSQGSPALYRN
Bi-WDSPSQGDPALYRN
Bi-WDSPAQGpSPALYRN
E.
Cdc27
+ CK2 + [γ-32P]-ATP
Bi-WDDDSAApSAAAAA
Bi-WLSPTISpSPPNALP
Bi-WLSPTISSPPNALP
Bi-WLSPTISDPPNALP
Bi-WLSPAISpSPPNALP
F.
Wee1A
+ CK2 + [γ-32P]-ATP
Bi-WDDDSAApSAAAAA
Bi-WGFGSSpSPVKAPA
Bi-WGFGSSSPVKAPA
Bi-WGFGSSDPVKAPA
Bi-WGFGASpSPVKAPA

Figure 4.1 Candidate peptides from a PhosphoSite search for S-X-X-S-P are not ideal substrates for primed CK2 phosphorylation A. Sequence of amino acids 1343-1364 of Topoisomerase IIα. A known canonical CK2 site at T1343 is shown in green, as are two putative hierarchical sites at S1351 and T1358. Known Cdk1 sites at S1354 and S1361 are shown in red. B. Biotinylated peptides corresponding to each CK2 phosphorylation site were incubated with CK2 and [γ-^{32}P]-ATP, bound to a streptavidin membrane, and visualized by autoradiography. Phosphoacceptor residues or corresponding mutations are in red. C-F. Biotinylated peptides corresponding to potential Cdk1/CK2 hierarchical phosphorylation sites in the mitotic proteins Separase (C, amino acids 1120-1131), Repo-Man (D, amino acids 126-139), Cdc27 (E, amino acids 363-375), and Wee1A (F, amino acids 118-129) were incubated with CK2 and [γ-^{32}P]-ATP and visualized as in B.

phosphorylation may be unfavourable when the Cdk1 site is located at the CK2 +3 position.

Presence of a Cdk1 site at the +3 position drastically reduces primed CK2 phosphorylation

Initial studies with sequences derived from a phosphorylation database search failed to produce any appreciable Cdk1/CK2 hierarchical phosphorylation. Therefore, to gain further insight into the consensus requirements for Cdk1/CK2 hierarchical phosphorylation, we next examined whether the intervening residues at positions +1 and +2 had any effect on CK2 primed phosphorylation. To do this, we based our assay on the peptide WDDDSAApSPVKAA, and changed either the +1 or +2 amino acid to arginine, phenylalanine, methionine, glutamine, histidine, phosphoserine, proline, or aspartic acid. Since aspartic acid itself is a consensus determinant for canonical CK2 phosphorylation, an additional peptide was included that contained the aspartic acid substitution in the presence of an unphosphorylated proline-directed serine. By comparing these two peptides, the impact of canonical phosphorylation can be separated from that of primed phosphorylation. Upon incubation with CK2 and [γ^{32}P]-ATP, however, even the alanine containing control peptide showed minimal phosphorylation by CK2 (Figure 4.2A,B). This was striking, as the only previously described peptide for primed CK2 phosphorylation, RRREEESAApSAA, contained the same determinants, yet lacked the CDK1 consensus site contained in the peptides in this study (23). Indeed, the only peptides that display increased primed CK2 phosphorylation in this assay were those with phosphoserine at either the +1 or +2 position, indicating that, like in the canonical consensus, multiple phosphorylated residues may be required for appreciable CK2

Figure 4.2

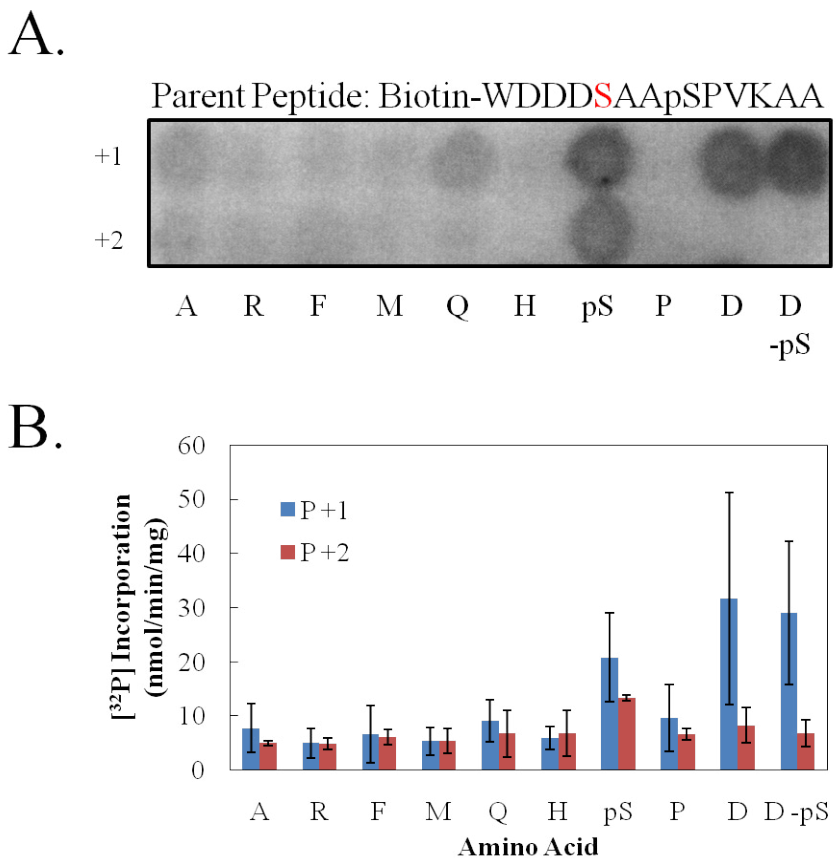

Figure 4.2 Influence of intervening residues on Cdk1/CK2 hierarchical consensus sequence. A. Starting from the parent peptide Bi-WDDDSAApSPVKAA (the phosphoacceptor residue is shown in red), the amino acid residues present at the +1 and +2 positions were individually changed to R, F, M, Q, H, pS, P, or D. To control for the ability of aspartic acid to act as a consensus determinant for CK2, an additional peptide with an unphosphorylated serine at the priming site was also included (denoted D-pS). Peptides were incubated with CK2 and [γ-^{32}P]-ATP, bound to streptavidin membrane, and [γ-^{32}P] incorporation was visualized. B. Average [γ-^{32}P] incorporation for each peptide after incubation with CK2. Values are an average of three independent experiments. Error bars indicate one standard deviation from the mean.

phosphorylation. Aspartic acid at the +1 position increased phosphorylation substantially; however this seems to be solely due to the formation of a canonical CK2 site, as the unphosphorylated control peptide showed similar levels of [γ^{32}P] incorporation. In accordance with the known canonical requirements, aspartic acid at the +2 position did not increase CK2 phosphorylation. Interestingly, glutamine, an uncharged hydrophilic amino acid, slightly but reproducibly increased phosphorylation when present at the +1 position. The utility of glutamine in a CK2 consensus sequence has never been reported before, but it may explain why the only candidate substrate tested above to display any evidence of CK2 primed phosphorylation was Repo-Man (Sequence: S-Q-G-pS-P-A-L) (See Figure 4.1D).

While this work does supply some clues on the consensus sequence for CK2 primed phosphorylation, the lack of kinase activity towards the peptides in this study indicates that the +3 position, while optimal for canonical CK2 phosphorylation, may not be optimal for Cdk1/CK2 hierarchical phosphorylation, as it seems that the presence of a Cdk1 consensus site inhibits CK2 phosphorylation. To confirm this, we compared the phosphorylation of peptides containing identical consensus determinants with either a string of alanines (AAAAA) or a Cdk1 consensus site (PVKAA) following the consensus phosphoserines. These peptides clearly show that the Cdk1 consensus determinants severely impairs the ability of CK2 to phosphorylate primed sequences when present at the +4 to +6 positions relative to the CK2 site (Figure 4.3A,B).

Impact of multiple phosphoserines and their order on CK2 primed phosphorylation

These results indicate that consensus determinants for primed phosphorylation by CK2 may differ from the consensus determinants for canonical phosphorylation.

Figure 4.3

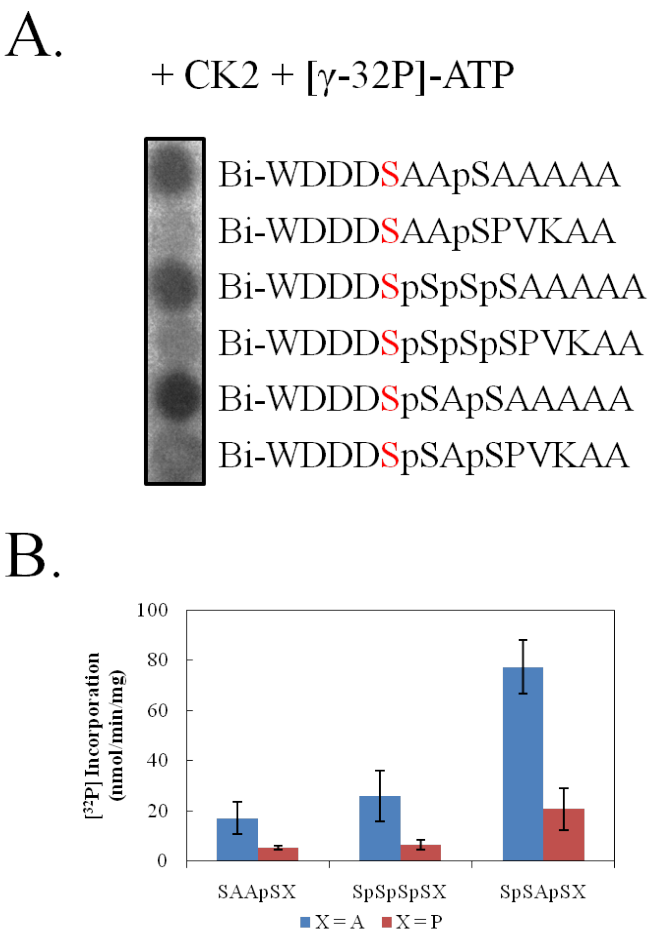

Figure 4.3 The presence of a Cdk1 consensus sequence at the +3 position dramatically decreases primed CK2 phosphorylation. Biotinylated peptides with either a Cdk1 consensus sequence or a string of alanines following a +3 phosphoserine were incubated with CK2 and [γ-^{32}P]-ATP, spotted on a streptavidin membrane and visualized by autoradiography. [γ-^{32}P] incorporation was visualized on a storm phosphorimager. The phosphoacceptor residue in these sequences is shown in red. Bi-, Biotin. B. Average [γ-^{32}P] incorporation into each peptide shown in A. Each value is an average of three independent experiments. Error bars indicate one standard deviation from the mean.

Therefore, before revisiting the prospect of hierarchical signalling, we elected to systematically examine the consensus determinants for primed phosphorylation. To determine the optimal spacing required between the CK2 phosphoacceptor residue and the priming phosphoserine, we devised an experiment based on the parent peptide WDDDSpSpSpSpSpSAAA, and systematically changed phosphoserine residues to alanine residues in all possible combinations. This experiment also gave us the opportunity to examine the effects of multiple phosphorylated residues on primed CK2 phosphorylation. As mentioned above, many canonical CK2 substrates contain several acidic residues C-terminal to the phosphorylation site (29). It was expected that this would hold true for primed substrates as well, with each additional phosphoserine adding to the acidity of the substrate. However, this was not the case. As shown in Figure 4.4, the level of CK2 phosphorylation among these substrates varied widely, even among peptides with the same total amount of phosphoserine. In fact, phosphorylation of the parent peptide (SpSpSpSpSpS) was barely detectable, and removal of almost any phosphoserine improved CK2 phosphorylation. The lack of phosphorylation of highly phosphorylated peptides suggests that there may be a threshold level of negativity required for CK2 phosphorylation, above which phosphorylation is actually discouraged. Indeed, changing additional phosphoserines to alanine increased phosphorylation by CK2 considerably, but only in certain cases. In peptides with 2-3 phosphoserine residues, the ability of CK2 to phosphorylate different combinations varied wildly, indicating that, like in the canonical consensus sequence, the precise positioning of phosphoserine is important for effective phosphorylation of a primed CK2 consensus sequence. It appears that, in general, the optimal number of phosphoserines for primed phosphorylation is 2-3,

Figure 4.4

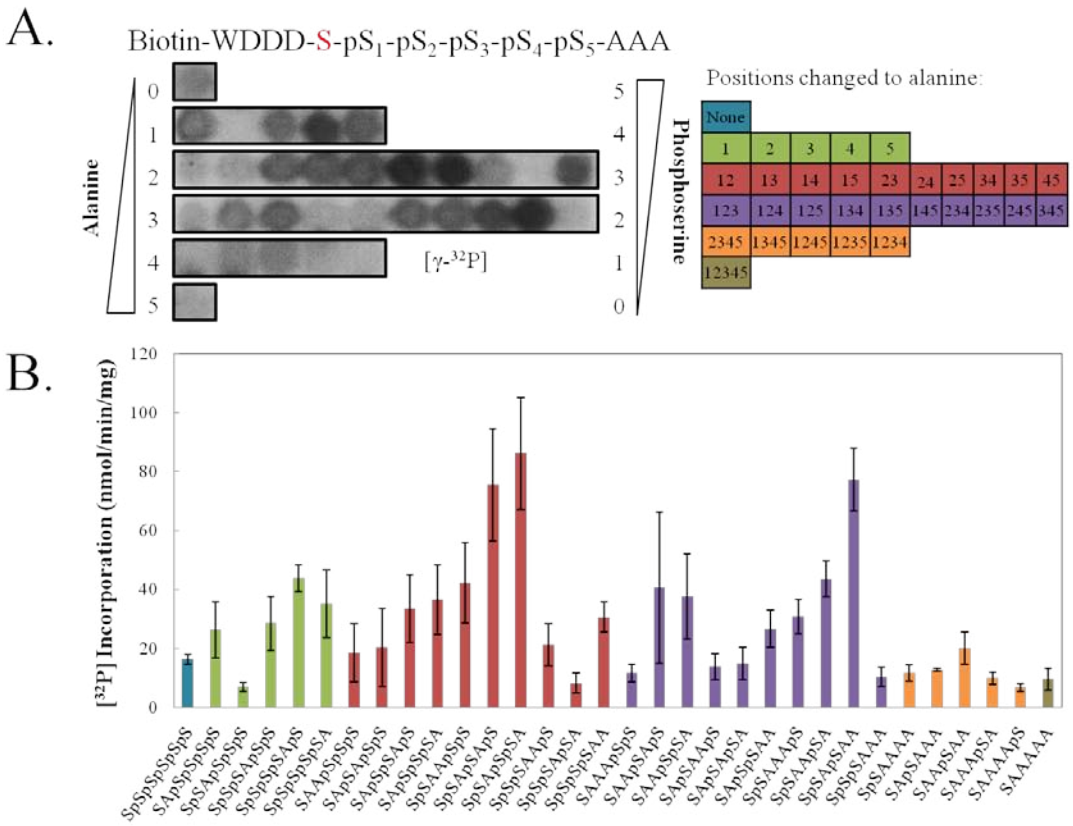
A.
Biotin-WDDD-S-pS1-pS2-pS3-pS4-pS5-AAA
Alanine
0
1
2
3
4
5
[γ-32P]
5
4
3
2
1
0
Phosphoserine
Positions changed to alanine:
None
1 2 3 4 5
12 13 14 15 23 24 25 34 35 45
123 124 125 134 135 145 234 235 245 345
2345 1345 1245 1235 1234
12345
B.
[32P] Incorporation (nmol/min/mg)
120
100
80
60
40
20
0
SpSpSpSpSpS
SApSpSpSpS
SpSApSpSpS
SpSpSpSpSAp
SpSpSpSpSpA
SAApSpSpS
SApSApSpS
SApSpSApS
SApSpSpSA
SpSAApSpS
SpSApSpSA
SpSpSAApS
SpSpSApSA
SpSpSpSAA
SAApSpSpA
SApSpSpSA
SApSpSApS
SApSpSAAp
SApSpSpAA
SpSAApSpA
SpSApSpAA
SpSpSAApA
SpSpSpAAA
SpSAApSAA
SpSApSpAA
SpSpSAAAA
SpSAAAA
SApSAAA
SAApSAA
SAAApSA
SAAAApS
SAAAAA
C.
Strong
+1 +2 +3 +4 +5
pS A pS pS A
pS A pS A pS
pS A pS A A
Medium
+1 +2 +3 +4 +5
pS pS pS A pS
pS pS pS pS A
A pS pS A pS
A pS pS pS A
pS A A pS pS
A A pS A pS
A A pS pS A
pS A A pS A
Weak
+1 +2 +3 +4 +5
pS pS pS pS pS
A pS pS pS pS
pS pS A pS pS
A A pS pS pS
A pS A pS pS
pS pS A A pS
pS pS pS A A
A pS pS A A
pS A A A pS
A A pS A A
Undetectable
+1 +2 +3 +4 +5
pS A pS pS pS
pS pS A pS A
A A A pS pS
A pS A A pS
A pS A pS A
pS pS A A A
pS A A A A
A pS A A A
A A A pS A
A A A A pS
A A A A A

Figure 4.4 Influence of quantity and spacing of phosphoserine residues on CK2 primed phosphorylation. A. Biotinylated peptides based on the sequence Bi-WDDDSpSpSpSpSpSAAA were incubated with CK2 and [γ-^{32}P]-ATP, spotted on a streptavidin membrane and visualized by autoradiography. A map of each peptide and the specific positions where phosphoserine was changed to alanine is also shown. B. Average [γ-^{32}P] incorporation into each peptide shown in A. Each value is an average of three independent experiments. Error bars indicate one standard deviation from the mean. C. Peptide phosphodeterminant sequences, grouped by relative phosphorylation of each peptide by CK2. Sequences are colour-coded by amount of phosphoserine as in the map in panel A.

as the only peptide containing just one phosphoserine to be detectably phosphorylated was the previously published primed sequence, SAApSAA.

When the peptides are sorted into groups based on their phosphorylation levels, distinct patterns start to emerge (Figure 4.4C). Three peptides in the study displayed strong phosphorylation by CK2: SpSApSpSA, SpSApSApS, and SpSApSAA. From this, we conclude that the optimal consensus sequence for primed CK2 phosphorylation will have phosphoserines at +1, +3, and possibly an additional phosphoserine at either +4 or +5. As is the case for canonical phosphorylation, primed phosphorylation by CK2 seems to be almost completely dependent on phosphodeterminants at the +1 and +3 positions. In fact, all detectably phosphorylated peptides in this study had at least one phosphoserine at either the +1 or +3 position, and of the 11 peptides that remained unphosphorylated after incubation with CK2, only 4 peptides contained a phosphoserine at either the +1 or +3 positions. In addition to the +1 and +3 positions, additional acidity at the +4 or +5 positions typically increased CK2 phosphorylation, provided that the apparent threshold of negativity was not passed. In contrast, presence of a phosphoserine residue at the +2 position had at best a negligible effect on CK2 phosphorylation, and in many cases was actually inhibitory. For example, the SpSpSpSAA peptide displays only weak phosphorylation by CK2, but with an alanine instead of the +2 phosphoserine, the SpSApSAA peptide was one of the strongest peptides in the study. Acidic determinants at the +2 position are typically negligible for canonical signalling as well, but the inhibitory effects seem to be unique to primed phosphorylation. The obvious exception to this trend was the SpSApSpSpS peptide, which was curiously not detectably phosphorylated, even

though the rest of the peptides with four phosphoserines showed increased phosphorylation over the parent peptide.

Enzymatic comparison of canonical and primed CK2 consensus sequences

To examine the relative likelihood of canonical and primed phosphorylation by CK2, we next performed kinetic studies to compare the enzymatic efficiency of CK2 on a selection of peptide substrates. We selected several primed peptides, including both strongly and weakly phosphorylated substrates, and compared them to both optimized canonical CK2 substrates (WDDDSDDDDDAAA and WEEESEEEEEAAA) as well as a known canonical mitotic substrate for CK2 phosphorylation, T1343 of Topoisomerase IIα As shown in Figure 4.5, when an equal amount of each peptide (0.5 mM) is incubated with CK2 and [γ-^{32}P]-ATP, the strongest of the primed substrate peptides are phosphorylated by CK2 at comparable levels to the optimal canonical sequence (SDDDDD) and the known mitotic substrate (TopoII T1343), and at much higher levels than the previously published primed peptide (SAApSAA) (23). This suggests that primed substrates of CK2 may be as efficiently phosphorylated by CK2 as canonical substrates. To examine this further, we performed a kinetic analysis for each detectably phosphorylated peptide in Figure 4.5. The SAAAAA peptide was also included in the analysis, but kinetic measurement was not possible due to absence of phosphorylation. The results of this analysis are shown in Table 4.3. Km values of all three peptides modelled after previously published peptides (SDDDDD, SEEEEE, and SAApSAA) are similar to but lower than previously published Km values (0.06 mM, 0.18 mM, and 0.57 mM respectively) (22, 23). The optimized canonical substrate (SDDDDD) was by far the best peptide in the study, with a Vmax/Km ratio (a measure of phosphorylation

Figure 4.5

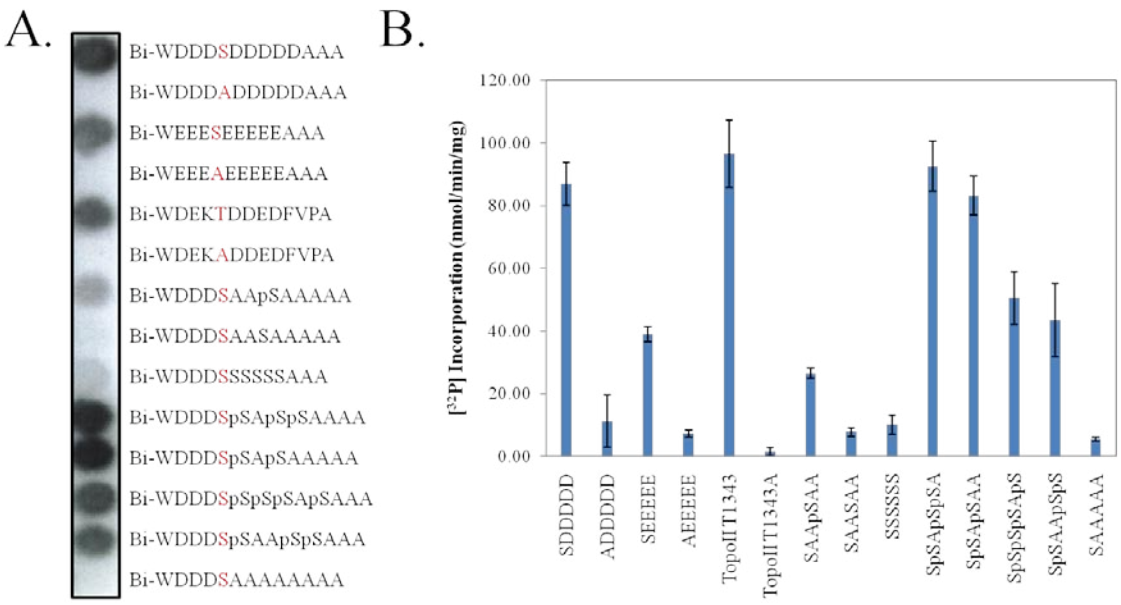

Figure 4.5 Relative comparison of canonical and primed phosphorylation by CK2. A. N-terminally biotinylated synthetic peptides conforming to both canonical (acidic) and primed (previously phosphorylated) CK2 consensus sequences were incubated with CK2 and [γ-^{32}P]-ATP before spotting onto streptavidin-coated membrane. The phosphoacceptor residue in these sequences is shown in red. [γ-^{32}P] incorporation was visualized on a Storm phosphorimager. Bi-, Biotin. B. Relative phosphorylation of synthetic peptides shown in A. Values are an average of three independent experiments. Error bars indicate one standard deviation from the mean.

TABLE 4-3. Kinetic constants for phosphorylation of CK2 canonical and primed consensus peptides

Peptide	Vmax (μmol/min/mg)	Km (mM)	Vmax/Km	SE_{Vmax}	SE_{Km}
WDDDSDDDDDAAA	0.609	0.017	36.37	0.022	0.003
WEEESEEEEEAAA	0.293	0.046	6.38	0.022	0.013
WDEKTDDEDFVPA	0.142	0.060	2.36	0.024	0.045
WDDDSAApSAAAAA	0.069	0.275	0.25	0.006	0.069
WDDDSpSApSpSAAAA	0.276	0.116	2.38	0.019	0.029
WDDDSpSApSAAAAA	0.183	0.091	2.01	0.017	0.031
WDDDSpSpSpSApSAAA	0.130	0.231	0.56	0.011	0.050
WDDDSpSAApSpSAAA	0.096	0.076	1.26	0.006	0.01

Kinetic constants were determined as described in section 4.2. pS, phosphoserine; SE, standard error.

efficiency) of 36.37, compared to 6.38 for the SEEEEE peptide (demonstrating the difference in efficiency between aspartic acid and glutamic acid as determinants for phosphorylation), and 2.38 for the highest efficiency primed peptide (SpSApSpSA). However, the peptide representing a known canonical mitotic substrate of CK2, T1343 of Topoisomerase IIα, despite having a lower Km value than any of the primed substrates, provided a Vmax/Km value of 2.36, equal to that of the SpSApSpSA peptide. Since T1343 of Topoisomerase IIα is quite effectively phosphorylated by CK2 during mitosis (7), this suggests that given the correct primed sequence, CK2 may be just as likely to phosphorylate a primed substrate as a canonical substrate.

Among the primed substrate peptides, it appears that both too many and too few phosphoserines are detrimental to CK2 phosphorylation efficiency, as the peptides with one phosphoserine (SAApSAA) and four phosphoserines (SpSpSpSApS) have higher Km values than the peptides with two or three phosphoserines. This correlates well with the results in Figure 4.4, where in general the highest CK2 activity was with diphosphopeptides and triphosphopeptides. The Km values of the most efficient primed substrates (SpSApSpSA, SpSApSAA, and SpSAApSpS) were similar (0.116 mM, 0.091 mM and 0.076 mM respectively), and the relative efficiency of phosphorylation between these samples seemed to depend more on their Vmax values. Due to this, the primed substrate with the lowest Km value (SpSAApSpS; 0.076 mM) was the least efficient substrate of the three, as the Vmax value for this peptide was two- to threefold lower than the others. These results indicate that it may be crucial to have the optimal number of phosphoserines in the correct spacing in order for a primed substrate to have reaction kinetics as favourable as a canonical substrate.

CK2 primed phosphorylation requires serine as both phosphodeterminant and phosphoacceptor

In previous work on primed phosphorylation by CK2, is was shown that while CK2 can use phosphoserine as an acidic determinant, it cannot use phosphothreonine in this manner (23). However, this study was done with peptides containing only one phosphodeterminant (RRREEESAApSA, RRREEESAApTA), which in our results show only weak phosphorylation. Given the difference in phosphorylation level between our low efficiency (SAApSAA) and high efficiency (SpSApSpSA) primed substrates, we chose to revisit the question of which phosphoamino acids can act as determinants for CK2 phosphorylation. Phosphotyrosine has also been demonstrated to act as a phosphodeterminant in CK2 hierarchical signalling (27), so it was included in our study as well. To compare phosphorylation of peptides containing each type of phosphodeterminant, we generated peptides corresponding to one of the most highly phosphorylated primed peptides (SpSApSpSA) with phosphoserine substituted with either phosphotyrosine or phosphothreonine. After incubation with CK2 and [γ-^{32}P]-ATP only phosphoserine proved useful as a phosphodeterminant, however, with no detectable phosphorylation with phosphothreonine and very little with phosphotyrosine (Figure 4.6A). Addtionally, we examined whether there was a difference in phosphorylation if the serine in the CK2 phosphoacceptor site is replaced with threonine. As shown in Figure 4.6B, substitution of serine with threonine drastically decreased phosphorylation by CK2, consistent with previous work on canonical phosphorylation (22). These results show that optimal CK2 primed phosphorylation requires serine as both the phosphoacceptor and phosphodeterminant residues.

Figure 4.6

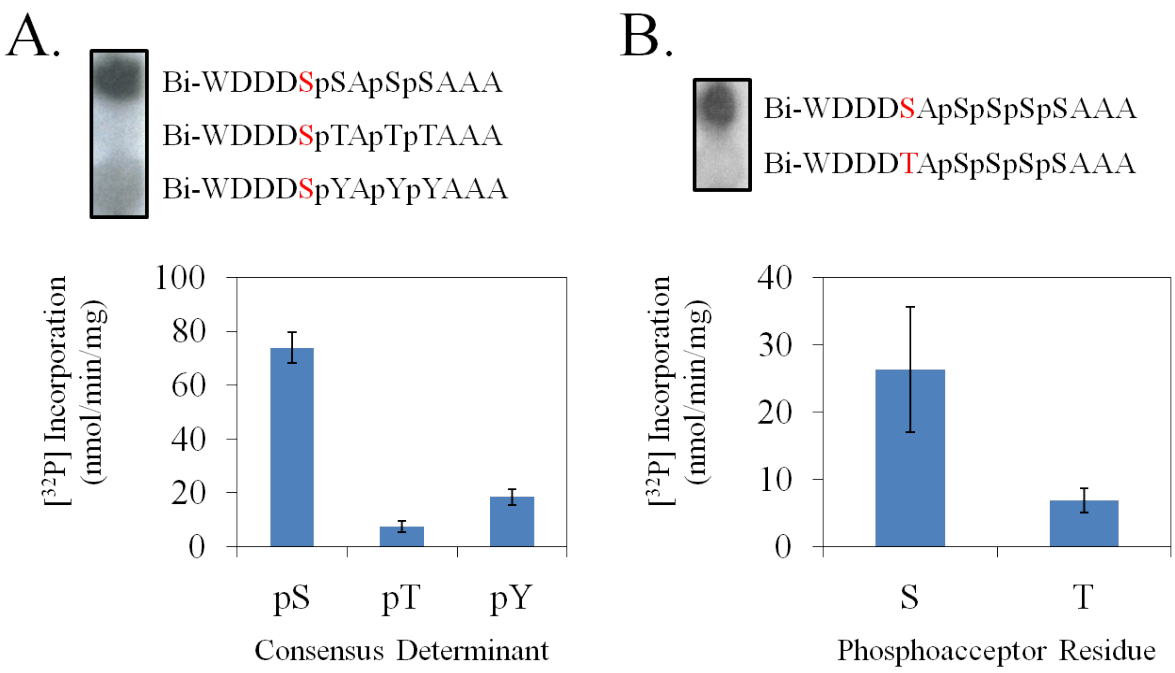

Figure 4.6 Primed CK2 phosphorylation favours serine as the phosphodeterminant and phosphoacceptor. A. Primed peptides with phosphoserine, phosphothreonine, or phosphotyrosine as the phosphodeterminants were incubated with CK2 and [γ-^{32}P]-ATP before spotting onto streptavidin-coated membrane. The phosphoacceptor residue in these sequences is shown in red. [γ-^{32}P] incorporation was visualized on a Storm phosphorimager. Bi-, Biotin. Values are an average of three independent experiments. Error bars indicate one standard deviation from the mean. B. Primed phosphopeptides with either serine or threonine in the phosphoacceptor site were incubated with CK2 and [γ-^{32}P]-ATP and processed as in panel A.

Cdk1 phosphorylation can synergize with canonical determinants to form a novel CK2 phosphorylation site

Collectively, these results outline the various factors controlling primed phosphorylation by CK2, and will be useful in identifying potential substrates within the genome. However, while hyperphosphorylated regions like those required for primed CK2 phosphorylation are widely distributed in the genome (41), with respect to hierarchical phosphorylation between Cdk1 and CK2, presumably only one serine residue will be phosphorylated at any given potential site (excluding the possible effects of additional kinases). While in most cases one phosphoserine residue is not sufficient to enable primed phosphorylation by CK2 (Figure 4.4), we wondered if Cdk1 phosphorylation could synergize with existing canonical determinants, providing enough additional acidity to the region to form a novel CK2 site. Alternatively, Cdk1 phosphorylation downstream of an existing weak CK2 site could strengthen the affinity of CK2 for that site, leading to increased phosphorylation. The results in Figure 4.3 indicate that Cdk1 consensus determinants following a phosphoserine at the +3 position drastically decreases the ability of CK2 to phosphorylate primed sites. In fact, the only highly phosphorylated peptide containing this sequence contained an aspartic acid at the +1 position (Figure 4.2). However, this was due to canonical phosphorylation, as a non-primed control peptide was also effectively phosphorylated by CK2. Since aspartic acid is a much stronger determinant for CK2 phosphorylation than glutamic acid (22) (also compare SDDDDD and SEEEEE peptides in Figure 4.5 and Table 4.3), we used glutamic acid to investigate the effects of addition of a Cdk1-catalyzed phosphoserine to a weak canonical CK2 site. To do this, we generated biotinylated peptides with a Cdk1

site phosphoserine at +3, +4, or +5, and glutamic acid at either the +1 or +3 position, and tested the phosphorylation of these peptides by CK2. By comparing peptides with phosphorylated and unphosphorylated Cdk1 sites, we could compare the baseline efficiency of the canonical determinants with any added effect from the Cdk1 phosphorylation event. As shown in Figure 4.7, the peptides containing one glutamic acid and an unphosphorylated Cdk1 site were barely phosphorylated, indicating that these sequences represent very weak CK2 sites. With glutamic acid at the +1 position, phosphorylation of the Cdk1 site at any position had little to no effect on CK2 phosphorylation of the sequence (Figure 4.7, panels A-C). However, when glutamic acid was included as an acidic determinant at the +3 position, phosphorylation of a Cdk1 site at either the +4 or +5 position resulted in a significant increase in CK2 phosphorylation compared to the unphosphorylated peptide (Figure 4.7, panels D and E). A Cdk1 site situated at the +5 position was particularly favourable. In this study, we also compared primed versions of each peptide with either alanine residues or a Cdk1 consensus sequence following the ultimate phosphoserine, and interestingly, while all other primed peptide pairs tested in this study demonstrated a sharp decrease in phosphorylation upon addition of the Cdk1 determinants (Figure 4.3), peptides with the Cdk1 site at the +5 position actually displayed increased phosphorylation compared to a corresponding peptide with alanine residues in place of the Cdk1 site (Figure 4.7, panels A and D). This suggests that, while unfavourable when directly adjacent to the CK2 site, the prolyl, hydrophobic, and basic determinants comprising a Cdk1 consensus sequence may actually become favourable determinants for CK2 phosphorylation when situated farther

Figure 4.7

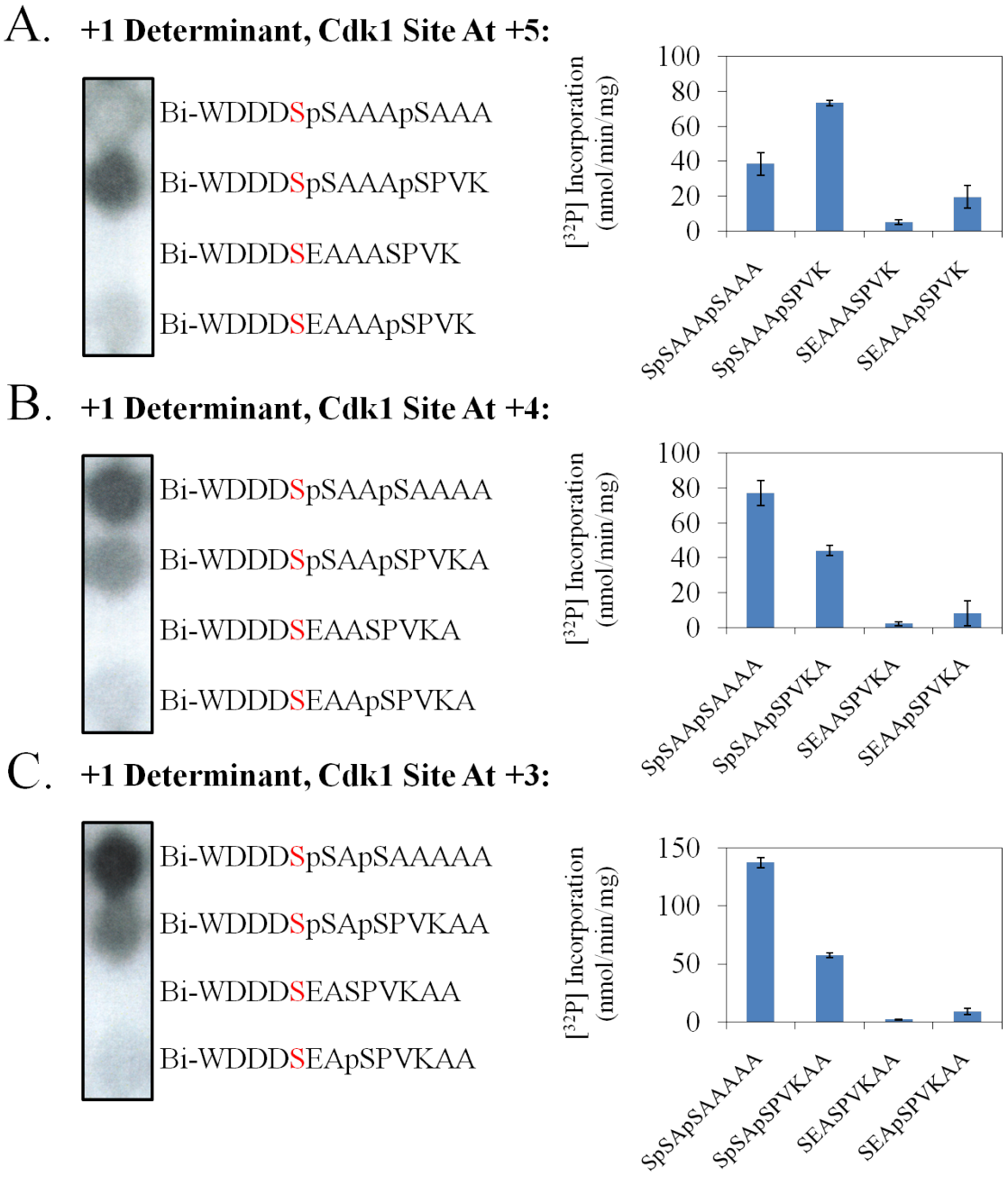
A. +1 Determinant, Cdk1 Site At +5:
Bi-WDDDSpSAAApSAAA
Bi-WDDDSpSAAApSPVK
Bi-WDDDSEAAASPVK
Bi-WDDDSEAAApSPVK
[32P] Incorporation (nmol/min/mg)
100
80
60
40
20
0
SpSAAApSAAA
SpSAAApSPVK
SEAAASPVK
SEAAApSPVK
B. +1 Determinant, Cdk1 Site At +4:
Bi-WDDDSpSAApSAAAA
Bi-WDDDSpSAApSPVKA
Bi-WDDDSEAASPVKA
Bi-WDDDSEAApSPVKA
[32P] Incorporation (nmol/min/mg)
100
80
60
40
20
0
SpSAApSAAAA
SpSAApSPVKA
SEAASPVKA
SEAApSPVKA
C. +1 Determinant, Cdk1 Site At +3:
Bi-WDDDSpSApSAAAAA
Bi-WDDDSpSApSPVKAA
Bi-WDDDSEASPVKAA
Bi-WDDDSEApSPVKAA
[32P] Incorporation (nmol/min/mg)
150
100
50
0
SpSApSAAAAA
SpSApSPVKAA
SEASPVKAA
SEApSPVKAA

Figure 4.7 Continued

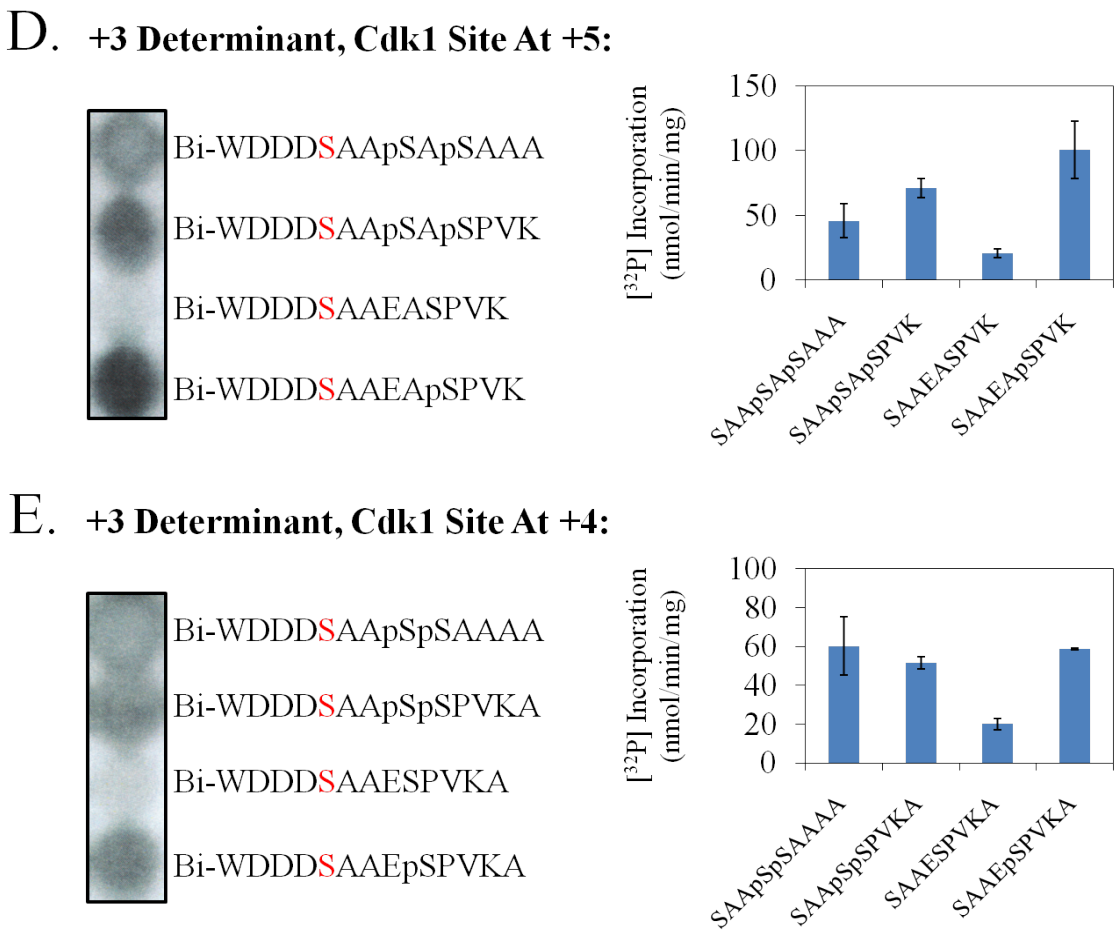

Figure 4.7 Cdk1 phosphorylation can form a novel site for CK2 phosphorylation. Biotinylated peptides corresponding to various spacing options for Cdk1/CK2 hierarchical phosphorylation were incubated with CK2 and [γ-^{32}P]-ATP before spotting onto streptavidin-coated membrane. [γ-^{32}P] incorporation was visualized on a Storm phosphorimager. The phosphoacceptor residue in these sequences is shown in red. Bi-, Biotin. Graphs represent [γ-^{32}P] incorporation into each peptide. Values are an average of three independent experiments. Error bars indicate one standard deviation from the mean.

away from the CK2 phosphoacceptor residue. Based on these results, we conclude that hierarchical phosphorylation between Cdk1 and CK2 requires both canonical and primed consensus determinants, and occurs optimally at sequences with a weak canonical determinant at the +3 position followed by a Cdk1 phosphorylation site at the +5 position (S-X-X-E-X-pS-P-V-K).

Genome-wide search for potential substrates of Cdk/CK2 hierarchical phosphorylation

Knowing the optimal spacing for Cdk1/CK2 hierarchical phosphorylation, we next wanted to search the human genome for protein sequences matching this spacing. However, while in the above experiment a glutamic acid at the +3 position was sufficient for hierarchical phosphorylation, given the flexibility of the CK2 consensus determinants it was necessary to include other options for CK2 consensus determinants. To do this, we searched for proteins in the human genome containing the sequences S-X-X-X-X-S-P, producing a dataset independent of both CK2 consensus determinants and particular Cdk consensus motifs. Due to this, the resultant dataset theoretically included all possible sites for hierarchical phosphorylation between any proline-directed kinase and any primed kinase. This allows additional filtering of the dataset based on the particular primed kinase and proline-directed kinase of interest. The total dataset is included as a table in Appendix A. To sort the results based on the positive and negative CK2 determinants found at positions 2-5 in the sequence, we implemented a scoring system as follows: The presence of serine at position 1 of the sequence scores 3.8, which acts as a baseline score. Since acidic residues are positive determinants for CK2 phosphorylation, especially at the +1 and +3 positions (29), presence of aspartic acid or glutamic acid were considered extremely favourable (+1.5) at any position. Previous work has demonstrated that proline,

lysine, and arginine are all negative determinants of CK2 phosphorylation when present at the +1 position (26, 28), so the presence of these residues at position 2 of the above sequence was considered extremely unfavourable (-1.5). Since our results suggest that non-charged hydrophilic residues like glutamine may aid in CK2 primed phosphorylation, we considered glutamine, asparagine, serine or threonine at positions 2-5 to be somewhat favourable as well (+1). All other residues at these positions were scored as neutral. The results were sorted by score into several groups: Sequences with strong, intermediate, and weak canonical determinants, and sequences with either no canonical determinants or negative determinants. While completely arbitrary numerically, the scoring system worked surprisingly well, with strong candidates for CK2 phosphorylation consistently scoring the highest score, 5.8. In general, intermediate and weak candidates for phosphorylation tended to score 5.3 and 4.8, respectively. Poor candidates for CK2 phosphorylation, with scores ranging from -0.5 to 4.8, were excluded from further study. Of the 7376 results returned by the peptide match program, 1262 of them contained weak, intermediate, or strong canonical determinants. All potential CK2 substrates in the dataset are included in a table in Appendix A.

To examine the types of genes potentially affected by hierarchical phosphorylation between CK2 and proline-directed kinases, we next used Gene Ontology (GO) analysis to determine the molecular functions of the peptide match results and the biological processes in which they participate. GO analysis compares the composition of our peptide match dataset against the human genome to identify associated biological processes and molecular functions that are statistically enriched in our dataset compared to the genome (16). A list of all genes included in GO analysis is included as a table in

Appendix A. Generally, the dataset was highly enriched for the molecular function "phosphoprotein" (58% of peptide matches; P = $5.6E10^{-45}$), though perhaps not surprisingly, as each peptide match contains at least two serine residues. Clustering of the genes in the dataset into functionally related classifications using high stringency parameters produced 16 separate gene clusters that were significantly enhanced in proteins containing a potential hierarchical phosphorylation motif, including centrosomal proteins, microtubule motor proteins, several GTPase regulatory proteins, and several classes of transcription factors (Table 4.4), indicating that several distinct groups of proteins are enriched in the peptide match results. When the genes were functionally annotated into groups based on biological process and molecular function GO terms, proteins containing hierarchical phosphorylation consensus determinants tended to cluster into distinct groups. Using the highest stringency settings, the most significantly enriched biological process amongst the genes in our dataset was "negative regulation of microtubule depolymerization', with an enrichment score (the mean of all P values for enrichment for all genes in the cluster) of 4.87 (E > 1.3 is considered significant), and a P value of $1.2E10^{-5}$ (Table 4.5A). With such strict stringency settings, only eight significant clusters were formed from the dataset, indicating that proteins containing possible proline-directed/CK2 hierarchical phosphorylation sites are concentrated into specific pathways, including those controlling microtubule dynamics, induction of apoptosis, and development of specific body tissues. This trend was accentuated further when annotation stringency was lowered to the high setting, thereby including a larger proportion of the dataset. Significant clustering results show that proteins containing potential proline-directed/CK2 hierarchical phosphorylation sites are enriched in pathways controlling

transcription, chromatin regulation, cell death, specific developmental and differentiation pathways, and, with respect to mitosis, the regulation of mitotic spindle dynamics.

Potential candidates for Cdk/CK2 hierarchical phosphorylation on the mitotic spindle

As our original goal was to investigate whether Cdk1 and CK2 could work together to phosphorylate mitotic proteins, thereby contributing to the regulation of mitosis, we next mined both the peptide match dataset and the resultant GO terms for proteins with known roles in mitosis. All proteins in the dataset listed under the GO terms "Microtubule cytoskeleton", "Cell division and chromosome partitioning", and "Regulation of mitotic cell cycle" were included. The resultant list contains 74 potential phosphorylation sites in 71 proteins (the microtubule-associated proteins MAP1B and MAP4 each contain two potential sites, as does the nucleolar protein Nucleophosmin) (Table 4.6). As the most enriched group of genes in the dataset was those involved in microtubule dynamics, the vast majority of mitotic candidates for hierarchical phosphorylation are components of the mitotic spindle. Validation of these candidates as potential substrates for Cdk1/CK2 hierarchical phosphorylation will undoubtedly increase our understanding of mitotic regulation, and determine the extent to which hierarchical phosphorylation contributes.

TABLE 4.4 Enriched gene function classifications in SXXXXSP peptide match results.

Cluster	Gene Function	Number of Genes	Enrichment score[a]	Fold Enrichment	P value[b]
1	Nucleolar Proteins	7	10.80	16	2.60E-08
2	Centrosomal Proteins	9	7.10	44	3.60E-12
3	Transcription factors (Zinc-finger)	67	6.91	5.3	5.90E-26
4	Transcription factors (Basic leucine zipper)	6	6.73	260	2.70E-13
5	Negative regulators of transcription	12	6.00	27	6.10E-17
6	Positive regulators of transcription	6	5.78	24	5.30E-08
7	Regulation of Rab GTPase activation	5	5.67	230	1.30E-10
8	Positive regulators of transcription	7	5.14	17	7.20E-07
9	WD40 Repeat proteins	12	4.50	57	1.40E-20
10	Microtubule motor proteins	12	4.28	98	6.30E-14
11	3'5'-cyclic nucleotide phosphodiesterases	6	4.16	660	1.90E-15
12[c]	Small GTPase regulation	8	4.04	230	7.80E-18
12[c]	Positive regulators of apoptosis	8	4.04	37	3.90E-12
13	Helicases	7	3.54	79	1.40E-12
14	Ubiquitin Peptidases	11	3.39	250	2.00E-25
15	Transcription Factors (Homeobox)	5	3.24	80	9.50E-09
16	Protein Kinases	30	2.19	27	2.10E-29

[a] The enrichment score represents the geometric mean of all the enrichment P values from all genes in the cluster. E > 1.3 is considered significant.
[b] The P value represents the significance of gene-term enrichment in this study. A P value of < 0.05 indicates significant enrichment in this dataset compared to the human genome.
[c] Genes in Cluster 12 had two functional classifications.

TABLE 4.5 Functional annotation analysis of SXXXXSP peptide match results.

A. Highest Stringency

Cluster	Representative GO Term	# of Genes	E[a]	Fold Enrichment	P value[b]
1	Negative regulation of microtubule depolymerization	7	4.87	12.23	1.19E-05
2	Regulation of microtubule depolymerization/ repolymerization	8	3.34	7.21	8.85E-05
3	3'5'-cyclic nucleotide phosphodiesterase activity	7	2.77	7.79	2.01E-04
4	Histone methyltransferase activity	7	2.33	4.79	3.03E-03
5	Embryonic limb morphogenesis	9	1.67	2.89	1.24E-02
6	Positive regulation of apoptosis	25	1.65	1.63	2.10E-02
7	Digestive tract morphogenesis	4	1.35	5.32	3.73E-02
8	Regulation of apoptosis	39	1.28	1.36	4.80E-02

B. High Stringency

Cluster	Representative GO Term	# of Genes	E[a]	Fold Enrichment	P value[b]
1	Regulation of gene expression	159	8.28	1.56	1.30E-09
2	GTPase regulator activity	44	7.17	2.91	6.06E-10
3	Biological regulation	310	4.88	1.20	8.11E-05
4	Chromatin modification	27	4.42	2.75	5.77E-06
5	Negative regulation of microtubule depolymerization	7	4.10	12.22	1.19E-05
6	Negative regulation of cytoskeleton organization	11	4.07	5.59	2.12E-05
7	Transcription	124	4.02	1.65	7.36E-09
8	Transcription from RNA Polymerase II promoter	20	3.33	2.39	7.52E-04

TABLE 4.5 continued

Cluster	Representative GO Term	# of Genes	E	Fold Enrichment	P value
9	Negative regulation of gene expression	36	3.10	2.00	1.36E-04
10	Positive regulation of transcription	38	3.06	1.88	2.69E-04
11	3'5'-cyclic nucleotide phosphodiesterase activity	7	2.77	7.79	2.01E-04
12	Negative regulation of transcription	32	2.62	1.95	5.20E-04
13	Immune system development	21	2.40	1.94	2.21E-03
14	GTPase binding	13	2.38	3.18	7.63E-04
15	Apoptosis	35	2.09	1.63	5.60E-03
16	Stem cell differentiation	6	2.04	5.24	5.10E-03
17	Microtubule motor activity	10	1.90	3.46	2.20E-03
18	Histone methyltransferase activity	7	1.89	4.79	3.00E-03
19	Embryonic limb morphogenesis	9	1.67	2.89	1.20E-02
20	Positive regulation of apoptosis	25	1.65	1.63	2.10E-02
21	Regulation of neuron differentiation	14	1.60	2.52	8.10E-03
22	Metal ion binding	178	1.39	1.13	3.74E-02
23	Digestive tract morphogenesis	4	1.34	6.58	2.10E-02

[a] The enrichment score represents the geometric mean of all the enrichment P values from all genes in the cluster. E > 1.3 is considered significant.

[b] The P value represents the significance of gene-term enrichment in this study. A P value of < 0.05 indicates significant enrichment in this dataset compared to the human genome.

TABLE 4.6 Potential mitotic spindle substrates for Cdk/CK2 hierarchical phosphorylation.

Gene Name	Position	Sequence	CK2 Score
5-azacytidine induced 1	8	traigSVPERSPagv	4.8
Actin filament associated protein 1-like 2	207	llcykSSKDHSPqld	5.3
Amyloid beta (A4) precursor protein-binding, family B, member 2	265	swttlSQDSASPssp	4.8
B-cell CLL/lymphoma 6	460	gsprsSSESHSPlym	4.8
Brain-enriched guanylate kinase-associated homolog	547	dslepSSMEASPemh	5.3
Budding uninhibited by benzimidazoles 1 homolog beta (yeast)	568	sesitSNEDVSPdvc	5.3
CAP-GLY domain containing linker protein 2	36	aavaaSSKEGSPlhk	5.3
Caspase recruitment domain family, member 11	477	eaddsSTSEESPeds	5.3
CDK5 regulatory subunit associated protein 2	47	llpnvSEETVSPtra	5.3
Centromere protein J	725	rergiSSREDSPqvc	5.3
Centrosomal protein 164kDa	1232	slsseSSESFSPphr	4.8
Centrosomal protein 350kDa	1647	ghhddSDEEASPekt	5.8
Centrosomal protein kizuna	617	seasfSSSEGSPlsr	5.3
Checkpoint with forkhead and ring finger domains	190	ptasaSSTEPSPagr	5.3
CHK1 checkpoint homolog	295	skhiqSNLDFSPvns	5.3
Ciliary rootlet coiled-coil, rootletin	1477	glnspSTLECSPgsq	5.3
Cortactin binding protein 2	1579	lrmpvSQKEVSPlss	5.3
CP110 protein	164	dlardSEGFNSPkqc	4.8
Cyclin B3	43	tkispSSLQESPssl	4.8
Cytoplasmic linker associated protein 1	759	dtsreSSRDTSParg	5.3
Cytoplasmic linker associated protein 2	768	easreSSRDTSPvrs	5.3
DAB2 interacting protein	28	rslpgSLSEKSPsme	4.8
Dedicator of cytokinesis protein 5	1507	evkqiSTEEISPlen	5.3
Dedicator of cytokinesis protein 6	1660	eesaiSDDILSPdee	4.8
Dedicator of cytokinesis protein 7	1720	eesavSDDVVSPdee	4.8
Dedicator of cytokinesis protein 8	1705	eesvvSEDTLSPded	5.3

TABLE 4.6 Continued.

Gene Name	Position	Sequence	CK2 Score
Dynein heavy chain domain 1	1628	ktiasSEPSLSPaac	5.3
Dynein, axonemal, heavy chain 5	2400	vfmssSILDWSPile	4.8
Dynein, axonemal, heavy chain 8	538	yffknSDILSSPdgk	4.8
Dynein, cytoplasmic 2, heavy chain 1	407	lknyiSEIQDSPqql	5.3
Farnesyltransferase, CAAX box, alpha	367	lqskhSTENDSPtnv	4.8
FYVE and coiled-coil domain containing 1	1244	sgsgtSQGEPSPals	5.3
Growth arrest-specific 2 like 1	600	qalssSSDEGSPcpg	5.3
Hepatocyte nuclear factor 4, alpha	13	gapveSSYDTSPseg	5.3
Intraflagellar transport 20 homolog	93	cklclSDSSDSPtsp	5.3
Kinesin family member 18B	458	megnsSDQEQSPede	5.8
Kinesin family member 1B	862	lpligSQEQKSPgsh	4.8
Kinesin family member 20A	861	tptcqSSTDCSPyar	5.3
Kinesin family member 21A	1265	sdsgtSEASLSPpss	5.3
Kinesin heavy chain member 2A	134	aqqngSVSDISPvqa	4.8
Kinesin light chain 3	496	rtlsaSTQDLSPh	5.3
Kinesin light chain 3	161	dppaeSQQSESPprr	4.8
Lysosomal trafficking regulator	1384	lkiieSDTTMSPsqy	5.3
MAP/microtubule affinity-regulating kinase 1	469	tvgskSEMTASPlvg	5.3
Microtubule-associated protein 1A	1197	dtqslSLSEESPske	4.8
Microtubule-associated protein 1B	1723	elisvSQVEASPsts	5.3
Microtubule-associated protein 1B	1959	ysydiSEKTTSPpev	5.3
Microtubule-associated protein 4	93	hgvegSDTTGSPtef	5.3
Microtubule-associated protein 4	199	lnsphSESFVSPeav	4.8
Microtubule-associated protein tau	226	rdvdeSSPQDSPpsk	4.8
M-Phase phosphoprotein	237	tfislSSTDVSPnqs	5.3
Myosin XVIIIB	108	ilgkeSEGSRSPdpe	5.3
NEDD8 activating enzyme E1 subunit 1	102	sdvsgSFVEESPenl	4.8
Neuron navigator 1	1259	klqhgSTETASPsik	4.8
Ninein-like	179	lqtwdSEDFGSPqks	4.8
Nucleophosmin/nucleoplasmin, 2	153	edadiSLEEQSPvkq	4.8
Nucleophosmin/nucleoplasmin, 2	190	eeiraSVRDKSPvkk	4.8
Pericentrin	48	savdaSVQEESPvtk	4.8
Phosphodiesterase 4D, cAMP-specific	196	lyrsdSDYDLSPksm	5.8

TABLE 4.6 Continued.

Gene Name	Position	Sequence	CK2 Score
Protein kinase, membrane associated tyrosine/threonine 1	88	frgeaSETLQSPgyd	4.8
PTPRF interacting protein, binding protein 1 (liprin beta 1)	354	daqgfSDLEKSPspt	5.8
RAB11 family interacting protein 3 (class II)	396	dygegSEAELSPetl	5.8
Rho/Rac guanine nucleotide exchange factor (GEF) 2	642	rglfrSESLESPrge	4.8
SMAD family member 6	316	apgefSDASMSPdat	5.3
Spectrin repeat containing, nuclear envelope 1	159	vdsivSSETPSPpsk	4.8
Sperm associated antigen 17	464	dlvppSLREPSPrad	4.8
Sperm associated antigen 4	100	vrggaSEPTGSPvvs	5.3
Synaptojanin 2	570	sgatdSQDDSSPadi	5.3
Syntrophin, beta 2	227	psfsgSEDSGSPkhq	5.3
Transformation/transcription domain-associated protein	2334	qailtSLIEKSPdak	4.8
TRIO and F-actin binding protein	131	nedpgSDPTSSPdsa	5.3
V-myc myelocytomatosis viral oncogene homolog	239	sdsllSSTESSPqgs	5.3
Zinc finger protein 655	279	ksceaSDKSCSPssg	5.3
Zinc finger, C3H1-type containing	122	rmpssSLSESSPrps	4.8

4.4 Discussion

In this study, we have examined the consensus requirements for phosphorylation of primed sequences by protein kinase CK2, in an effort to determine whether interplay between CK2 and the main mitotic regulatory kinase Cdk1 could contribute to mitotic signalling. The results of our study demonstrate that this may indeed be the case, as we have shown that phosphorylation of an adjacent Cdk1 consensus sequence can contribute to the formation of a novel site for CK2 phosphorylation. This indicates that CK2 kinase activity towards particular substrates can be regulated by the actions of proline-directed kinases. In a genome-wide search for sites matching the consensus sequence for hierarchical phosphorylation by proline-directed kinases and CK2, the mitotic spindle was particularly enriched with proteins containing putative consensus sequences.

This study is the first to systematically investigate CK2 primed phosphorylation, and the results indicate that in terms of positioning, primed consensus determinants tend to follow similar patterns as canonical determinants. For example, primed phosphorylation preferentially requires phosphodeterminants at the +1 and +3 positions. The similarities in spacing requirements are due to the geometry of the CK2 substrate binding cleft, which uses three distinct basic regions of the kinase to coordinate acidic determinants for phosphorylation into the active site, positioning the phosphoacceptor residue for catalysis (39). There are, however, several subtle differences in the optimal sequences for each type of phosphorylation. For example, while in canonical signalling the +2 position is negligible (39), in primed signalling we found that in most cases phosphodeterminants at the +2 position actually inhibited phosphorylation. Additionally, while a canonical site typically contains multiple acidic determinants (29), too many

phosphodeterminants actually inhibits CK2 phosphorylation of a substrate, and due to this there appears to be a limit on the acceptable number of phosphosites in a primed CK2 consensus sequence, at 2-3. This may be explained by the fact that while aspartic acid and glutamic acid, with pKa values of 3.9 and 4.07, respectively, will each bear a maximum of one negative charge at physiological pH, each phosphoryl group, with a pKa value of ~6.7, will most likely have two negative charges at physiological pH (18). Since binding of CK2 to its substrates appears to be largely dependent on negative charges in the substrate, we propose that CK2 sites may have an upper threshold of negativity, above which the high number of negative charges may interfere with substrate phosphorylation. This idea is supported by mutational studies performed on the basic regions of the CK2 substrate binding cleft, as mutation of certain basic residues to alanine results in decreased CK2 phosphorylation due to an increase in Km, meaning that CK2 cannot bind its substrates as effectively (39). This may also somewhat explain why phosphodeterminants at the +2 position actually decrease primed phosphorylation: the basic residues in the CK2 substrate binding pocket are optimally positioned to bind to acidic determinants at the +1 and +3 positions, but an additional two negative charges between these two binding sites may affect electrostatic interactions between the basic residues and proper acidic residues dramatically. When each determinant has one negative charge, an acidic residue at the +2 position is ineffectual; with each site containing two negative charges the binding pocket may be overwhelmed and bind the substrate incorrectly, leading to decreased phosphorylation. Due to this, it seems that the increased acidity of phosphoserine results in increased selectivity in the phosphorylation of primed substrates compared to canonical substrates. It is intriguing to speculate that

this enhanced emphasis on optimal positioning may act as a regulatory mechanism controlling these events. As sites of hyperphosphorylation are rampant in the human phosphoproteome (41), the strict requirements observed may ensure hierarchical phosphorylation by CK2 only at very specific layouts of phosphoserine residues. In addition, the increased acidity of phosphoserine means that residue to residue, less phosphoserine would be required than aspartic acid or glutamic acid to create a functional CK2 site. This would in fact increase the likelihood of primed phosphorylation *in vivo*, as CK2 would only require 2-3 phosphorylated residues, albeit with the correct spacing, in order to efficiently catalyze phosphorylation.

During the course of this study, we also conducted experiments to determine the preferences for different phosphoresidues as both phosphodeterminants and phosphoacceptors. As the human phosphoproteome is composed of approximately 86.4% phosphoserine, 11.8% phosphothreonine, and 1.8% phosphotyrosine (34), it is perhaps not surprising that predominantly phosphoserine residues are found in hierarchical phosphorylation events, but our results demonstrate that in fact only serine is favourable as both the phosphodeterminant for CK2 phosphorylation and as the CK2 phosphoacceptor residue. This suggests that hierarchical phosphorylation somehow evolved to involve only phosphoserine. The reasons behind this preference are probably due to subtle structural differences between phosphodeterminant residues that impact the ability to the substrate to properly fit in the CK2 substrate binding domain. In the case of phosphothreonine, the additional methyl group may skew the angle of the phosphate in relation to the remainder of the substrate, which may lead to decreased affinity for the CK2 substrate binding pocket. The lack of primed phosphorylation with

phosphothreonine as a determinant may be unique to CK2, as CK1, another primed kinase, can use both phosphoserine and phosphothreonine as phosphodeterminants (13). Conversely, while CK2 can and in most cases does utilize aspartic acid and glutamic acid as consensus determinants, CK1 cannot (13), indicating that the loss of phosphothreonine as a possible determinant for CK2 phosphorylation may be due to subtle differences in the substrate binding pocket of CK2 give it the unique ability to participate in both canonical and primed phosphorylation. In the case of phosphotyrosine, the increased length of phosphotyrosine compared to phosphoserine may impact the ability of the phosphodeterminant to fit in the binding pocket, although the impact is presumably less than that for phosphothreonine, as phosphotyrosine in the phosphodeterminant positions did lead to substrate phosphorylation, albeit at much lower levels than when phosphoserine is used as a phosphodeterminant. At the phosphoacceptor site, it is important to consider that CK2 tends to prefer serine over threonine in general, as do most serine/threonine kinases (37), and accordingly our results show that even with identical phosphodeterminants to a highly phosphorylated serine-containing peptide, phosphorylation of threonine was only barely detectable. It should also be noted that CK2 can in certain cases phosphorylate tyrosine residues (47), but in the context of primed phosphorylation, this remains to be investigated.

With respect to phosphorylation efficiency, the primed peptides that displayed maximal [γ-^{32}P] incorporation showed similar levels of phosphorylation to both the optimal canonical CK2 substrate peptide (SDDDDD) and a peptide based on the canonical consensus sequence of a known mitotic substrate of CK2, Topoisomerase IIα (T1343), indicating that given the correct sequence determinants, primed phosphorylation

by CK2 can be just as efficient as canonical phosphorylation. Kinetic analysis of canonical and primed peptides confirmed this, as the better primed substrates had quite respectable Km values, and exhibited a Km/Vmax ratio comparable to the Topoisomerase IIα mitotic phosphorylation site, suggesting that primed phosphorylation by CK2 may be as likely to occur in the cell as some canonical events.

Since CK2 prefers phosphodeterminants at the +3 position, we originally hypothesized that Cdk1/CK2 hierarchical phosphorylation sites would generally contain the Cdk1 phosphoserine at the CK2 +3 position (SXXSPVK). While a number of mitotic proteins contain similar motifs, our results indicate that hierarchical phosphorylation of these sequences is extremely unfavourable. This seems to be due to the close proximity of the prolyl, hydrophobic, and basic characteristics of the Cdk1 consensus sequence to the CK2 site, as similar peptides with alanine residues following the Cdk1 phosphoserine were phosphorylated quite well by CK2. This, combined with the observation that multiple phosphodeterminants are required for appreciable primed phosphorylation by CK2, makes it unlikely that these types of events would occur in a cellular context, at least not without the added contribution of other kinases, which indeed may be a possibility. The mitotic regulatory kinase Plk1 may be a likely candidate for these sorts of higher order phosphorylation events, as its phosphorylation determinants mirror those of CK2, with Plk1 requiring acidic residues N-terminal to the phosphosite (31), and Plk1 interacts with CK2 during mitosis via the mitotic phosphorylation sites (likely catalyzed by Cdk1) located in the C-terminus of CK2α (25). The interaction between Cdk1 and CK2 during mitosis could also aid in the phosphorylation of these events, with Cdk1 essentially targeting CK2 to these sites. Regardless, our results show that, at least in

peptide form, Cdk1/CK2 hierarchical phosphorylation is unfavourable when the Cdk1 site is the only phosphodeterminant downstream and extremely unfavourable when the Cdk1 consensus determinants are close to the CK2 site. In fact, during investigation of this possibility, even Wee1A, which was previously reported to undergo Cdk1/CK2 hierarchical phosphorylation *in vivo*, was not detectably phosphorylated by CK2 in our assay. This was initially worrisome, but further investigation revealed an additional known Cdk1 phosphorylation site at serine 127 of Wee1A (6), which may add enough additional acidity to the sequence to form an efficient CK2 site. As this site was not phosphorylated in our peptide (and in fact was changed to alanine to remove the possible effects of additional serine residues), we were unable to observe any effects it may have on phosphorylation of serine 121 by CK2. Even with this additional phosphoserine residue, however, the positioning of these sites remains unusual, as the sequence contains both a phosphoserine at the +2 position and a proline at the +3 position. However, there are instances of canonical CK2 substrates that do not match the typical consensus sequence (29), and it is possible that Wee1A may be the exception to the rule in terms of primed CK2 phosphorylation.

As our results suggest that purely primed hierarchical phosphorylation between Cdk1 and CK2 is unlikely, we next investigated the possibility of hierarchical phosphorylation between Cdk1 and CK2 in the context of mixed consensus determinants. To do this, we started with a weak canonical CK2 consensus motif (a single glutamic acid residue at either the +1 or +3 positions), and asked whether phosphorylation of a Cdk1 site downstream from the CK2 site could synergize with the existing weak CK2 consensus determinant. The results from this experiment were striking, and again

highlight the strict spacing requirements for efficient CK2 phosphorylation. We found that Cdk1 sites can indeed contribute to the formation of novel CK2 sites, as long as the Cdk1 site phosphoserine is located a minimum of four amino acids downstream from the CK2 site, and optimally five amino acids downstream. Interestingly, while the presence of Cdk1 consensus determinants drastically decreased CK2 phosphorylation when the Cdk1 phosphoserine was located at the +3 position, the presence of the same Cdk1 consensus determinants farther downstream actually increased CK2 primed phosphorylation compared to similar peptides containing alanines, suggesting that these residues may be favourable determinants for CK2 phosphorylation, provided they are positioned far enough away from the active site of CK2.

These results demonstrate that the phosphorylation of a Cdk1 site can, with the correct positioning, drastically increase phosphorylation of a weak canonical CK2 site. Accordingly, we were interested in determining if these sites existed in the genome, as in the cell this effect could have a dramatic impact on CK2 signalling, and may explain how CK2, while constitutively active, can be targeted to particular substrates in response to a specific stimulus. While our main goal was to specifically investigate the possibility of interplay between CK2 and Cdk1, we opted to expand our search to look for genomic instances of the sequence SXXXXSP. It is important to note that results theoretically represent hierarchical phosphorylation events between any proline-directed kinase and any primed kinase. The Cdk1 consensus sequence was removed from the search to avoid the loss of possible substrates due to atypical Cdk1 consensus determinants. We reasoned that the precise proline-directed kinase involved could be inferred for any given match based on the precise determinants present in the amino acid sequence and the cellular

properties of the gene product involved, for example its expression throughout the cell cycle. Also, it is much more likely that the proline-directed kinases acting on these sites would be cyclin-dependent kinases and not MAPKs, as MAPKs typically require an additional proline at the -2 position (31), which in our sequence lies at the +3 position with respect to the CK2 site, a position where proline would be extremely unfavourable. Similarly, the sequence used in the peptide match search does not match the optimal spacing of consensus determinants for phosphorylation by CK1 (pS/pTXXS)(13) or GSK3 (SXXXpS) (12), indicating that no other known primed kinase is likely to appreciably phosphorylate these sites. Additionally, we sorted the resultant peptide matches based on the presence of CK2 canonical determinants. The search for canonical determinants was conducted after the more generic peptide match search because while the experiment conducted demonstrated that a glutamic acid at the +3 position was sufficient to synergize with the phosphorylated Cdk1 site, CK2 canonical determinants can be much more flexible, and we wished to avoid missing any possible substrates containing other combinations of CK2 canonical determinants. To then isolate the promising CK2 substrates from the rest of the results, we devised a scoring matrix based on known CK2 consensus requirements. The scoring system allowed us to quickly exclude unfavourable sequences, and organize the peptides containing CK2 determinants into three groups based on the strength of the putative CK2 sites in each result. It is expected that, when tested, genes in the different groups will vary in their initial phosphorylation status and may also show varied effects after addition of the Cdk phosphoserine. Indeed, many of the results in the group with the strongest consensus

determinants may be sufficient to form canonical phosphorylation sites that are indifferent to downstream phosphorylation.

GO analysis of the peptide match dataset produced several distinct and highly significant clusters, indicating that instances of hierarchical phosphorylation between CK2 and proline-directed kinases may occur specifically in certain biological pathways. Intriguingly, the predominant biological processes found to be enriched in our peptide match results overlap well with the known functions of cyclin-dependent kinases, the two most well studied being the regulation of cell cycle progression and the regulation of transcription (10).

As the goal of this study was to examine possible interplay between Cdk1 and CK2, we were encouraged by the extremely significant enrichment of specific families of mitotic genes in the peptide match results. In particular, the most significant biological function enriched in the study was ‘negative regulation of microtubule depolymerization’, and the genes clustered into this group included a myriad of structural components of the mitotic spindle, including several microtubule binding proteins (MAPs), centrosomal components, and kinetochore components, as well as several ATP-driven motor proteins (Kinesins, Dynein). Additional peptide match results included several mitotic regulatory proteins, including the known Spindle Assembly Checkpoint (SAC) component Bub1 (32), CHFR, a relatively uncharacterized checkpoint protein that localizes to the mitotic spindle and may act as a sensor for microtubule disruption (3), and Chk1, a well known component of the G1/S and G2/M DNA damage checkpoints (38) that has recently been implicated in SAC signalling as well (5, 36). Taken together, this suggests that Cdk1/CK2 hierarchical phosphorylation may play a role in the regulation of microtubule

dynamics, potentially as part of the SAC. Several lines of evidence suggest that CK2 could be a vital component of SAC signalling, which acts at the metaphase to anaphase transition to ensure that all chromosomes are properly attached to the mitotic spindle before initiation of sister chromatid separation at the onset of anaphase (32). CK2 has been shown to localize to the mitotic spindle and centrosomes (21, 50), and proteomic investigations have identified CK2 as a component of both the centrosome (1) and the spindle midbody (42). Localization of CK2 to the mitotic spindle is dependent on the mitotic phosphorylation of the CK2α catalytic subunit (see Chapter 3), which is thought to be catalyzed by Cdk1 (2, 24, 43). These sites, while phosphorylated during normal mitosis, are maximally phosphorylated in response to treatment with mitotic spindle poisons like nocodazole (2, 24), indicating a role for CK2 in the SAC. Indeed, disruption of these phosphorylation sites results in multiple defects in mitosis, including centrosomal amplification, induction of mitotic catastrophe, and abrogation of the SAC (43). Additionally, attenuation of CK2 activity through chemical inhibitors or knockdown of CK2 subunits has also been demonstrated to neutralize the actions of the SAC (40). The validation of our peptide match results will hopefully shed new light on the role of CK2 in the SAC, particularly in the context of Cdk1/CK2 hierarchical phosphorylation.

While there was enrichment of several kinds of proteins involved in mitosis in our peptide match results, we did not see a corresponding enrichment in proteins involved in the G1/S transition, in which both Cdks and CK2 play active roles (10, 44). Interestingly, as with MAPKs, two of the main G1/S regulatory Cdks, Cdk4 and Cdk6, tend to require an additional proline at the -2 position (31). Additionally, although CK2 interacts directly with the Cdk-activating kinase (CAK) complex, which includes Cdk7, and is responsible

for activation of the G1/S Cdks following growth signalling, the consensus determinants for Cdk7 phosphorylation, which include basic residues at the -3 and -4 positions (31), would presumably interfere with Cdk7/CK2 hierarchical phosphorylation. It is tempting to speculate that this may in part explain the lack of enrichment of G1/S biological processes in the peptide match results.

Besides cell cycle related processes, the overwhelming majority of the enrichment in the peptide match results stemmed from genes involved in transcriptional control, particularly transcription from RNA polymerase II-driven promoters. The results indicated that our dataset contains multiple enriched clusters of different families of transcription factors, as well as separate clusters containing both positive and negative regulators of transcription. Interestingly, Cdks (mainly Cdk7, Cdk8, and Cdk9) have been shown to regulate transcription both directly, through the phosphorylation of both the RNA polymerase II C terminal domain (CTD) and various transcription factors, as well as indirectly through interactions with both activators and suppressors of transcription (11). CK2 is also a well-known regulator of transcription. Like Cdk1, it also directly regulates RNA polymerase II via CTD phosphorylation, and phosphorylates a variety of RNA polymerase II general transcription factors, including TFIIA, TFIIE, and TFIIF, leading to increased transcriptional activation (4). CK2 also phosphorylates a variety of other transcription factors, leading to either activation or repression of transcription, depending on the substrate (44). As Cdks and CK2 seem to have multiple, overlapping roles in the regulation of transcription, interplay between the two kinase families may have a significant effect on cellular gene expression.

In this study, we have examined the possibility of hierarchical phosphorylation between CK2 and Cdks, particularly Cdk1, and the results of this endeavour suggest that instances of interplay between the two kinases may have a significant impact on mitosis, and particularly the regulation of the mitotic spindle. Our future work will involve kinase assays with peptide arrays to assess promising substrates for hierarchical phosphorylation. To determine the impact of previous phosphorylation on CK2 phosphorylation at these sites, peptide arrays will contain peptides of each sequence in both the unphosphorylated and phosphorylated states. This will also allow us to judge the optimal strength of the initial CK2 site in each peptide, as presumably at least some of the stronger CK2 candidates will be canonical CK2 sites, and phosphorylation at these sites may increase with the addition of the phosphodeterminant or may remain unchanged. By comparing any trends observed between peptides with strong, intermediate and weak initial CK2 sites, we may be able to gain insight into the consensus requirements for these mixed phosphorylation events. Interesting candidates will then be assayed for *in vitro* phosphorylation of the full length protein, as phosphorylation of peptide substrates does not eliminate the possibility of these sites being inaccessible to kinases when contained in a folded protein. The use of full length proteins will require successive phosphorylation assays with first Cdk1 and then CK2. Lastly, it remains to be seen whether these events occur in cells, and if so, what impact on cellular signalling hierarchical phosphorylation will have. We plan to investigate this by expressing phosphorylation site mutants of promising candidates in human cells and assaying the effects of expression on substrate function and mitotic progression. Through this work, we hope to demonstrate that Cdk1/CK2 hierarchical phosphorylation contributes to mitotic signalling, and may in part

explain how a constitutively active kinase such as CK2 can play such specific roles in a multitude of cellular processes.

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Chapter 5: Summary and Perspectives

5.1 Overview

Mitosis is a highly regulated process that ensures the proper separation of chromosomes to the daughter cells, avoiding aneuploidy and genomic instability. As highlighted in this thesis, protein kinase CK2, and in particular the CK2α catalytic subunit, is crucial for proper cell division, but as CK2 is not subject to the strict, on/off forms of regulation often observed with other kinases, its participation in highly regulated processes implies that CK2 is regulated through more subtle means. Therefore, we hypothesized that CK2 is differentially regulated during mitosis, enabling CK2 to perform unique functions specific to cell division. First, we sought to examine the effects of mitotic phosphorylation of CK2α on mitotic progression. Although it has been long known that CK2α is maximally phosphorylated in cells arrested in mitosis by nocodazole treatment (3, 19), and that these phosphorylation sites form an interaction site for the peptidyl-prolyl isomerase Pin1 (27), the mechanism by which the phosphorylation of CK2α contributes to mitotic division has remained largely unknown. We have demonstrated that perturbation of these sites leads to multiple defects in cell division, indicating that proper phosphorylation of CK2α is crucial for normal cell division. Second, we have examined the subcellular localization of CK2α during mitosis, and showed that phosphorylation of CK2α leads to its localization to the mitotic spindle. This localization is dependent on the interaction between phosphorylated CK2α and Pin1, and could potentially target CK2 to important mitotic substrates. Lastly, to examine the possibility that interplay with the master mitotic regulatory kinase, Cdk1, may regulate CK2 activity during mitosis, we have outlined the consensus requirements for

hierarchical phosphorylation between the two kinases. We then used this consensus to predict several possible substrates, particularly among components of the mitotic spindle. Taken together, this thesis outlines multiple mechanisms by which CK2 may be regulated in order to fulfill its mitotic functions, including phosphorylation, subcellular localization, and interplay with other kinases. The data in this thesis and the implications of these findings are discussed in the following sections.

5.2 Regulation of CK2 through phosphorylation of CK2α

5.2.1 CK2α phosphorylation in mitosis

Upon entrance into mitosis, the cell undergoes a massive wave of phosphorylation, potentially altering the function of a myriad of enzymes (34). For the majority of proteins known to be phosphorylated during mitosis, the function of this phosphorylation remains unknown. The work in this thesis provides some vital clues towards determining the roles of the mitotic phosphorylation of CK2. The generation and characterization of phosphospecific antibodies against the CK2α C-terminal phosphorylation sites proved to be instrumental, allowing us to examine the temporal and spatial regulation of these phosphorylation events in a way that was not previously possible. These antibodies allowed us to demonstrate for the first time that CK2α is phosphorylated during the normal progression of mitotic cell division, not just in cells arrested in mitosis by activation of the spindle assembly checkpoint (SAC). We determined that in normal mitosis, CK2α is phosphorylated during prophase and metaphase, and dephosphorylated during anaphase. This corresponds well with the idea that Cdk1 is the kinase responsible for CK2α phosphorylation (3, 19), as phosphorylation of CK2α temporally corresponded with the activation of Cdk1. Additionally, the

phosphospecific antibodies allowed us to specifically visualize phosphorylated CK2α in mitotic cells for the first time. This lead to the observation that phosphorylated CK2α, but not unphosphorylated CK2α, localizes to the mitotic spindle during mitosis. This work provides an explanation for the phosphorylation of CK2α during mitosis: the C-terminus of CK2α is phosphorylated in order to localize a subset of cellular CK2 to the mitotic spindle during its formation and organization. Previous work in our laboratory had demonstrated that the peptidyl-prolyl isomerase Pin1, another enzyme known to be important in mitosis (24), can interact with CK2α through a phosphodependent interaction with the CK2α C-terminus (27), and our work suggests that the purpose of this interaction is to localize phosphorylated CK2α to the mitotic spindle. Unfortunately, due to lack of assays for Pin1 activity, it was not possible to determine whether Pin1 enzymatically alters the CK2α phosphorylation sites, somehow resulting in CK2 localization (possibly through additional protein-protein interactions), or whether Pin1 itself directly localizes CK2 to the mitotic spindle. In fact, the observation that Pin1 itself localizes to the mitotic spindle indicates that direct localization is certainly a possibility. However, a recently discovered phosphodependent interaction between the CK2α C-terminus and the mitotic kinase Plk1 (22) indicates that Pin1 may not be the only protein affecting the localization of CK2, as Plk1 also localizes to the mitotic spindle (10). While questions remain, the work in this thesis has expanded our understanding of the mitotic phosphorylation of CK2α and its role during cell cycle progression.

5.2.2 Disruption of CK2α phosphorylation in cells

The use of phosphospecific antibodies provided a wealth of information on mitotic CK2α phosphorylation, but in order to examine what possible effect these sites

have on mitotic progression, we also studied the effects of disrupting the CK2α phosphorylation sites. Accordingly, we developed stable cell lines with tetracycline-regulated expression of a bidirectional plasmid expressing both HA-tagged CK2α and Myc-tagged CK2β. This system has several advantages: first, the tetracycline regulation allows for comparison of one cell line in the presence and absence of tetracycline, eliminating the risk of clonal variation between different cell lines. Second, using a bidirectional plasmid to co-ordinately express both CK2α and CK2β maintains the stoichiometry of the tetrameric holoenzyme, minimizing possible effects of large amounts of free catalytic subunits. To study the impact of the CK2α phosphorylation sites on mitotic progression, we used cell lines expressing wild type CK2α, two phosphomimetic forms (CK2α-4D, CK2α-4E), or a nonphosphorylatable form (CK2α-4A). These cell lines allowed us to examine several aspects of cellular function: growth curves indicated decreased proliferation upon expression of all three phosphorylation site mutants, particularly the phosphomimetic mutants; cell cycle analysis revealed that expression of phosphomimetic CK2α lead to a loss of mitotic cells, which was identified through viability assays and immunofluorescence staining as mitotic catastrophe, a form of cell death during mitosis. Cells expressing phosphomimetic forms of CK2α also displayed abnormal centrosomes, which lead to chromosomal missegregation and may have been the stimulus for induction of mitotic catastrophe in these cells. Notably, cells expressing non-phosphorylatable CK2α did not show these phenotypes, but did show decreased ability to arrest via the SAC upon nocodazole treatment. These results indicate that proper regulation of CK2α phosphorylation is crucial for proper mitotic progression.

While investigating the localization of phosphorylated CK2α, C-terminal fragments of CK2α containing the same phosphorylation site mutations were examined for their ability to localize to the mitotic spindle. Interestingly, we observed no spindle localization of any C-terminal mutant fragment, while the wild type C-terminal fragment localized to the spindle independently of the remainder of the protein. This result suggests that all the observed phenotypes may be due to lack of phosphorylation of a few particular substrates of CK2 on the mitotic spindle. Expression of the non-phosphorylatable form of CK2α leads to defects in the SAC, suggesting that one or more of the regulatory proteins involved in the SAC may potentially be a substrate of CK2. Indeed, the phosphorylation of CK2α was originally observed in cells treated with the mitotic spindle poison nocodazole (19), and work in this thesis demonstrates that CK2α phosphorylation is dramatically increased in cells with an activated SAC compared to normal mitotic cells, also consistent with a role for CK2 in the SAC. Activation of the SAC inhibits the activity of the Anaphase Promoting Complex /Cyclosome (APC/C), a ubiquitin ligase that promotes progression from metaphase to anaphase through the ubiquitination and targeted degradation of several key mitotic proteins, including Cyclin B (29). Inhibition of Cyclin B degradation results in prolonged activation of Cdk1, which would in turn increase the ratio of phosphorylated CK2α to unphosphorylated CK2α in the mitotic cell. This may lead to increased CK2 localization to the mitotic spindle (Figure 5.1). The effects of this increase in phosphorylated CK2α remain unelucidated, but if CK2 is indeed a component of SAC signalling, this would form a positive feedback loop, possibly maintaining cell cycle arrest until the spindle defect has been repaired.

Expression of CK2α phosphomimetic mutants caused more severe mitotic defects, but did not cause the SAC defect observed in cells expressing nonphosphorylatable CK2α. Intriguingly, this difference in phenotype occurs despite the observation that neither form can properly localize to the mitotic spindle. In fact, the lack of spindle localization, despite the presence of negative charges to mimic the phosphorylation sites, is easily explained by the fact that Pin1, which we found to be required for CK2α localization, has a much higher affinity for phosphoserine and phosphothreonine than aspartic acid or glutamic acid (52), and has been demonstrated to be incapable of binding to phosphomimetic CK2α (27). However, one would expect that lack of phosphomimetic CK2α on the spindle would lead to the same SAC defect, and this was not observed. It is possible that the more dramatic phenotypes observed upon phosphomimetic CK2α expression are dominant negative effects, as the centrosomal defects these cells display would most certainly warrant an activated SAC. It seems that the response of these cells is to bypass the SAC entirely and instead induce mitotic catastrophe, a form of apoptosis during mitosis that is believed to be induced in situations where continuing cell division would risk aneuploidy in the daughter cells (4). While the cellular signalling pathways involved in the induction and execution of mitotic catastrophe have not yet been delineated, it is likely that CK2 could play a role, given the mounting evidence that CK2 is an important regulator of apoptotic signalling. Overexpression of CK2 protects cells from drug-induced apoptosis (7, 12), and inhibition of CK2 sensitizes cancer cells to both

Figure 5.1

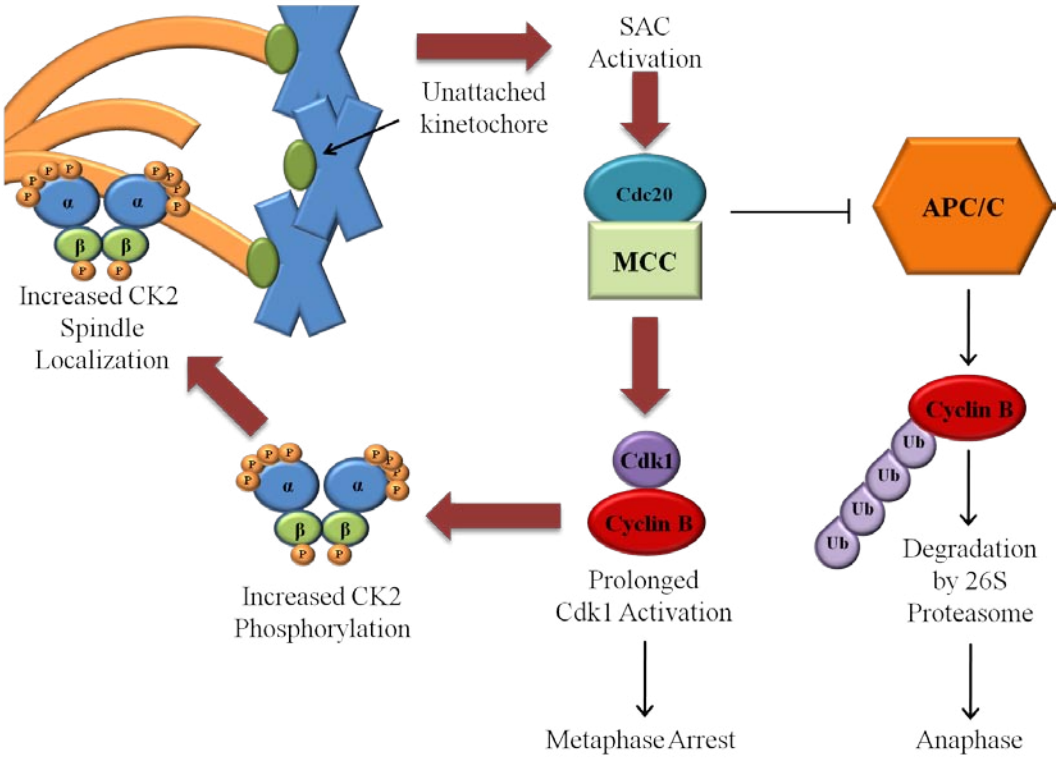

Figure 5.1 Model of the effects of spindle assembly checkpoint on the regulation of CK2. During metaphase, chromosomes attach to the microtubules of the mitotic spindle through a multiprotein complex called the kinetochore. The kinetochore also contains the components of the SAC, which monitors chromosome attachment. In the event of an unattached kinetochore, the SAC is activated, and the Mitotic Checkpoint Complex (MCC), composed of Mad2, BubR1, and Bub3, binds to Cdc20, a cofactor of the Anaphase Promoting Complex/Cyclosome (APC/C). Binding to Cdc20 inhibits activation of the APC/C, a ubiquitin ligase that targets Cyclin B for degradation by the 26S proteasome, enabling progression into anaphase. Inhibition of the APC/C by the SAC therefore leads to increased Cdk1 activation, which results in maximal CK2 phosphorylation and localization to the mitotic spindle. P, phosphorylated residue; Ub, ubiquitin.

receptor-mediated apoptosis (14, 15) and intracellular apoptosis in response to DNA damage (53, 54). CK2 phosphorylates a number of apoptotic regulatory proteins through a mechanism that, like hierarchical phosphorylation, exploits the unique consensus determinants of CK2 in order to affect widespread regulation of a biological process. In this case, the similarities between the acidic consensus requirements of CK2 and caspase cleavage motifs, which centre on an acidic aspartic acid residue, allows CK2 to phosphorylate residues within or near a caspase cleavage motif (18), blocking the access of the caspase to its substrate and protecting the substrate from cleavage (47). CK2 also directly regulates several caspases through phosphorylation, including caspase 9 (26) and caspase 3 (8). Furthermore, caspase 2, which is believed to be instrumental for induction of mitotic catastrophe (4), is also a CK2 substrate. Phosphorylation of caspase 2 by CK2 inhibits its ability to dimerize, thereby preventing its activation through autoproteolysis (44).

As mentioned above, the mitotic catastrophe observed in cells expressing phosphomimetic CK2α is correlated with centrosomal defects, suggesting that CK2 may play a role in the maintenance of centrosomal integrity. The centrosomes, which act as the microtubule organizing centres of mammalian cells, are replicated during S phase along with the chromosomes, and undergo several maturation stages before migrating to opposite poles of the cell to promote the formation of a bipolar mitotic spindle (6). Notably, CK2 localizes to the centrosomes (16), and proteomic investigation has confirmed that CK2 is a indeed a component of the centrosome (1). Additionally, the results of our peptide match search for proteins containing proline-directed sites for CK2 hierarchical phosphorylation were greatly enriched for centrosomal proteins, suggesting

that several novel centrosomal substrates of CK2 may exist. Intriguingly, both of the mitotic regulatory proteins known to bind specifically to phosphorylated CK2α, Plk1 and Pin1, have important regulatory roles in centrosome maturation and microtubule nucleation. Plk1 is a major regulator of centrosome maturation during prophase (6), and is required for proper bipolar spindle formation (48). Overexpression of Plk1 leads to centrosomal amplification (17), and conversely inhibition of Plk1 leads to monopolar spindles (10). Pin1 localizes to the centrosomes, and like Plk1 its overexpression causes centrosomal amplification (46). Interestingly, we observed that in cells with stable knockdown of Pin1, the centrosomes fail to separate; leading to monopolar spindles (see Figure 3.5, panel C). While this does not necessarily implicate CK2 in the effect, phosphorylated CK2α does not localize to the mitotic spindle in these cells, suggesting that the phenotype may be linked to lack of CK2 activity at the centrosomes. Not only do both enzymes bind the phosphorylated CK2α C-terminus, but Pin1 and Plk1 also interact directly in mitotic cells (43). Additionally, Plk1 participates in hierarchical phosphorylation with CK2 and Cdk1 (49), and Pin1 targets the proline-directed phosphorylation sites catalyzed by Cdk1 (52). The intricate interplay between these four important mitotic enzymes suggests the possibility of multienzyme complexes that could be targeted towards different functions. The presence of Pin1 in these complexes may play a role in enabling hierarchical phosphorylation between the three kinases, as prolyl isomerization of Cdk-phosphorylated sequences to the cis form renders the site resistant to dephosphorylation (45), potentially prolonging the phosphorylation at the site and enabling its use as a phosphodeterminant. Clearly, much more work is needed to delineate the roles of CK2 in mitosis, but the various phenotypes observed upon

disruption of mitotic phosphorylation of CK2α clearly indicate that CK2 is crucial for mitotic progression, and is potentially involved in multiple pathways ensuring proper cell division.

The fact that all of the phenotypes observed in cells expressing phosphorylation-site mutants of CK2α occurred without any obvious defects in kinase activity corresponds well with our later discovery that phosphorylation of CK2 modulates its localization to the mitotic spindle. Localization would regulate CK2 through targeting it towards certain mitotic substrates (and possibly also away from non-mitotic substrates), not through direct inhibition of CK2 kinase activity. Interestingly, our work in Chapter 4 on hierarchical phosphorylation by CK2 yielded an extensive list of potential CK2 substrates associated with the mitotic spindle, adding further evidence for an important role for CK2 at the mitotic spindle.

5.3 Regulation of CK2 through hierarchical phosphorylation

In an effort to understand how CK2 can be essentially constitutively active, and yet participate in multiple highly regulated processes, we chose to investigate whether interplay with other protein kinases could contribute to CK2 regulation. Given that most proteins are phosphorylated at multiple sites (33), and that an estimated 20% of the phosphoproteome can be attributed to CK2 alone (38), the interplay between CK2 and other kinases could have a substantial impact on the topology of the phosphoproteome. This may be particularly important during mitosis, when the majority of phosphorylation events depend, at least indirectly, on the activation of the mitotic master regulatory kinase, Cdk1 (34). Indeed, Wee1A represents a published example of hierarchical phosphorylation between Cdk1 and CK2 during mitosis (49). We therefore hypothesized

that hierarchical phosphorylation by Cdk1 and CK2 may be a widespread phenomenon during mitosis. An intriguing aspect of this idea is that in these cases, CK2 would essentially be under the same regulation of the priming kinase, in this case Cdk1, and this may explain how CK2 is targeted toward mitotic substrates.

5.3.1 Structural comparison of canonical and primed phosphorylation by CK2

CK2 is one of very few protein kinases that can utilize previously phosphorylated residues as consensus determinants to phosphorylate adjacent serine residues. However, this aspect of CK2 activity has not been systematically investigated. Accordingly, we performed a detailed investigation of the optimal consensus sequence for primed phosphorylation by CK2. This work found that, for the most part, primed phosphorylation requires similar consensus determinants as canonical phosphorylation, as positive determinants at the +1 and +3 positions relative to the phosphoacceptor site were particularly favourable, and multiple phosphoserine residues were optimal for efficient phosphorylation. However, we also observed some subtle differences, such as increased sensitivity to determinants in the +2 position, and an apparent increased reliance of optimal spacing for primed phosphorylation compared to canonical phosphorylation. Based on our knowledge of the mechanism by which CK2 binds its substrates, it is conceivable that these subtle differences are due to the increased negativity of phosphoserine, which is dianionic at physiological pH. While to date no structure of CK2 in complex with a peptide substrate has been solved, mutational studies have shown that coordination of a substrate in the CK2 catalytic cleft requires the concerted actions of three distinct basic regions in the sequence of the CK2 catalytic subunits (39, 40). These

basic residues are responsible for the proper coordination of acidic determinants on the substrate in the active site, allowing optimal positioning of the phosphoacceptor site for CK2 phosphorylation, and provide an explanation for the acidophilic nature of CK2. Additionally, the three groups of basic residues are responsible for the coordination of distinct determinants, with R191, R195, and K198 responsible for recognition of determinants at the +1 position, K79, R80 and K83 coordinating the +3 determinant, and K74-77 coordinating determinants at the +4 and +5 positions, as well as aiding in coordinating the +3 determinant (40). Therefore, CK2 consensus determinants are often found at these specific positions, even in canonical phosphorylation. This model of substrate binding is supported by a recently solved crystal structure of CK2α with two sulphate ions coordinated in the positions of the +1 and +3 determinants (30). However, binding of CK2 substrates in the catalytic cleft is flexible, as mutation of substrate determinants at the +1 and +3 positions can increase the importance of additional determinants at suboptimal positions (39, 40), indicating that to some extent, CK2 substrates can shift their positions in the binding pocket to optimally orient their phosphoacceptor residue, allowing suboptimal consensus sequences to be phosphorylated. However, while this seems to be the case for canonical substrates, the increased acidity of phosphoserine may restrict the flexibility of the binding cleft, and this may explain why optimal positioning of residues, particularly at the +1 and +3 positions, seems to be much more crucial for primed phosphorylation compared to canonical phosphorylation. Additionally, the increased sensitivity towards substrates with phosphoserine at the +2 position may also be explained by the increased negativity of primed substrates. The basic residues that accommodate acidic residues at the +1 and +3

positions could easily accommodate the dianionic phosphoserine in primed sequences (as they do the sulphate ions in the aforementioned CK2α crystal structure), as multiple positively charged residues exist at each site. However, the presence of an additional dianionic phosphoserine directly in between these sites may interfere with proper interactions between the +1 and +3 phosphoserines and their respective binding sites, inhibiting CK2 phosphorylation. It is intriguing to speculate that this would lead to increased specificity for primed phosphorylation compared to canonical phosphorylation, ensuring that primed phosphorylation by CK2 occurs only at specific sites.

5.3.2 CK2 hierarchical phosphorylation in mitosis

Since this study concerned the regulatory forces acting on CK2 during mitosis, we were particularly interested in determining the optimal consensus sequence for potential hierarchical phosphorylation events involving CK2 and the master mitotic regulatory kinase, Cdk1. Using the hierarchical phosphorylation of Wee1 as an example, we first attempted to investigate the optimal consensus sequence for direct primed phosphorylation events between Cdk1 and CK2, with the Cdk1 site at the +3 position relative to the CK2 phosphoacceptor sites. However, while several mitotic proteins contain these motifs, our results showed that these sequences were unsuitable for Cdk1/CK2 hierarchical phosphorylation. One reason for this is the presence of only a single phosphorylated residue at these sites, and the other is that presence of a Cdk1 consensus sequence was inhibitory when situated so close to the CK2 phosphoacceptor site. We next investigated whether phosphorylation of a Cdk1 site could synergize with weak CK2 canonical determinants to form novel sites for CK2 phosphorylation, and the results were striking, with a Cdk1 phosphoserine at the +5 position relative to the CK2

site enabling a dramatic increase in CK2 phosphorylation. This result suggests that there may be an entirely new list of potential substrates for CK2 among proteins with weak CK2 determinants adjacent to phosphorylation events catalyzed by other kinases. Strikingly, these sites would not be subjected to the constitutive activity displayed by CK2 towards the majority of its canonical substrates, but instead would be under the same regulatory constraints as the priming kinase. GO analysis of the results of our peptide match search showed that proteins containing sequences matching this pattern are highly enriched among centrosomal and mitotic spindle components, as well as several mitotic spindle regulatory proteins, including components of the SAC. These results indicate that phosphorylation of these 'conditional' CK2 substrates has the potential to dramatically affect the regulation of the mitotic spindle; consistent with our model that CK2α phosphorylation leads to its localization to the mitotic spindle, enabling phosphorylation of specific mitotic spindle substrates. The possibility of hierarchical phosphorylation of mitotic spindle substrates suggests that even once CK2 has localized to the mitotic spindle, additional regulatory forces (the activation states of priming kinases) ensure that CK2 phosphorylates its substrates with temporal precision throughout mitotic progression. While we examined interplay between Cdk1 and CK2, it is also possible that Plk1 phosphorylation could form novel CK2 sites, or lead to increased phosphorylation at existing CK2 sites. This is possible because the consensus determinants for Plk1 phosphorylation somewhat mirror the CK2 consensus determinants, requiring acidic residues N-terminal to the phosphoacceptor site (28). In this respect, for a CK2 site and a Plk1 site with shared acidic determinants, phosphorylation of the Plk1 site could increase the total acidity of the sequence,

promoting CK2 phosphorylation (Figure 5.2A). Plk1 is highly regulated throughout mitotic progression, and displays dynamic localization, localizing to the centrosomes during prophase, the kinetochores at the metaphase–anaphase transition, and the spindle midbody during cytokinesis (36). Consequently, the dynamic localization of Plk1 could temporally control CK2 phosphorylation at each of these sites. In fact, as some Plk1 sequences contain a proline following the acceptor site (28), it is likely that some of our peptide match results could represent Plk1/CK2 hierarchical phosphorylation events. It is also worth noting that acidophilic serine/threonine kinases like CK2 and Plk1 are rare in the human kinome, as the vast majority of human S/T kinases are basophilic (28), and therefore not amenable to participation in hierarchical phosphorylation with CK2. For example, the other main kinase family acting during mitosis, the Aurora kinases, are unlikely to prime substrates for CK2 phosphorylation, as the consensus requirements for these basophilic kinases would impede CK2 phosphorylation of nearby sequences (X-R-X-S for Aurora A; R-R-R-R-S for Auroras B and C) (28) (Figure 5.2B). This suggests that CK2 hierarchical phosphorylation may have evolved to allow interplay with only certain kinases.

5.3.3 Hierarchical phosphorylation as a signalling module

Our work on hierarchical phosphorylation also highlights the fact that as much as we know about individual phosphorylation events and their functional effects, a full understanding of the impact of phosphorylation on a given protein is impossible until we can identify the full complement of phosphorylation sites, the temporal regulation of the sites, and the interplay between them. Hierarchical phosphorylation is just one example

Figure 5.2

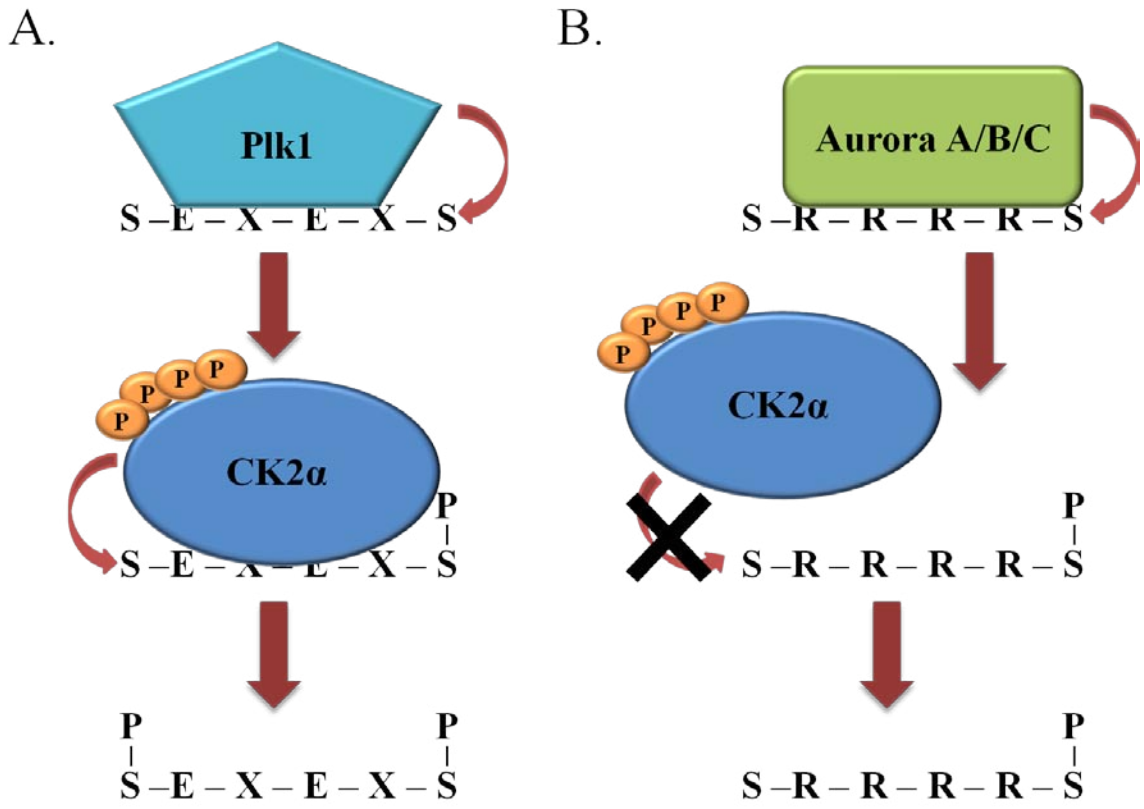

Figure 5.2 Potential for CK2 to participate in hierarchical phosphorylation with other major mitotic regulatory kinases. Based on the consensus determinants for phosphorylation by Plk1 (A) and Aurora kinases (B), Plk1 phosphorylation sites are favourable as sites for primed CK2 phosphorylation, as the Plk1 site could synergize with shared acidic determinants to either form a novel CK2 site or increase phosphorylation at an existing site. In contrast, Aurora kinases depend on N-terminal basic determinants to recognize and phosphorylate substrates, and these basic residues will inhibit CK2 phosphorylation of nearby phosphoacceptor sites. A single phosphorylated CK2α subunit is shown for simplicity. P, Phosphate.

of this interplay, and the first systematic study of the phenomenon is contained in this thesis. Ongoing efforts to compile accessible databases of *in vivo* phosphorylation sites will undoubtedly lead to increased understanding of the integration of various signals and pathways in the cell. In fact, analysis of existing phosphoproteomic data reveals that multisite phosphorylation is widespread in the human proteome. For example, in a study of 70,000 reported *in vivo* phosphorylation sites, a striking 54% of phosphoserine and phosphothreonine residues in the human phosphoproteome were found to be no farther than four amino acids away from another phosphoserine or phosphothreonine (41). An independent study of known phosphorylation sites in both mice and humans revealed a similar overabundance of phosphorylation sites that not only clustered into specific regions of substrates, but also seemed to be activated simultaneously (51). This clustering of sites indicates that the impact of processive and hierarchical phosphorylation on cellular signalling may be extensive, and potentially adds considerably to the already long list of potential CK2 substrates. The extreme clustering of phosphorylation sites observed in the phosphoproteome had led to the notion that combinations of phosphorylation sites may collectively act as higher order 'modules' that act as one to elicit a certain biological response (51). Mathematically, this idea has merit. A protein phosphorylated at N sites will exist in 2^N different phosphorylation states. For a protein with two phosphorylation sites, this equates to 2^2, or 4, different states, and it is entirely conceivable that these four states could all have distinct cellular functions. However, if each phosphorylation site on a protein elicited an independent functional response, a protein with 10 phosphorylation sites would exist in 2^{10}, or 1024 different phosphorylation states. It is highly unlikely that each of these states would be relevant biologically (37). Given the clustering of

phosphorylation sites and the apparent widespread use of hierarchical phosphorylation in the phosphoproteome, it is likely that a protein with 10 phosphorylation sites would in actuality have considerably less than 1024 different phosphorylation states. In this light, individual phosphorylation sites can be thought of as essentially building blocks, which together form various modules that can elicit different cellular responses. The functional effects of these modules may depend on the extent of phosphorylation within the region, as well as the pattern of the phosphorylation sites (37). Adding additional complexity, the interplay between phosphorylation sites and sites of other post-translational modifications, such as acetylation or SUMOylation, has not yet been systematically identified. Also important to delineate are the protein-protein interactions resulting from the modification of specific sites, including those involving the modular phospho-binding domains found in a multitude of signalling proteins. For example, in the Wee1 study that prompted our investigation, Cdk1 phosphorylation of serine 123 not only allows CK2 phosphorylation of serine 121, it also forms a docking site for the PBD of Plk1, resulting in phosphorylation of serine 53 by Plk1 (49). Clearly, the cataloguing of all posttranslational modifications and their interrelated effects on protein function represents a phenomenal amount of work, but only with a fully integrated knowledge of all the forces acting on a given protein following a given stimulus can we begin to fully understand the cellular functions of that protein. Our work outlining how CK2 uses phosphoserine as a determinant for phosphorylation will be vital to understanding how CK2 contributes to hierarchical phosphorylation in the cell.

5.4 Mechanisms for regulating CK2 activity in mitosis

The purpose of the work contained in this thesis was to gain a better understanding of the subtle regulatory forces acting on CK2 during mitosis. The presence of four mitotic phosphorylation sites on CK2α were our first indication that CK2, while constitutively active and involved in a myriad of cellular processes, may be differentially regulated during mitosis, enabling it to perform specific roles unique to mitosis. Indeed, these phosphorylation sites are merely the first step in the process of subverting a subset of cellular CK2 in order to enable cell division. Results in this thesis demonstrate that, once activated at the G2/M transition, Cdk1 phosphorylates a small fraction of the total CK2α in the cell. Phosphorylation leads to the formation of a phosphodependent binding site for Pin1, a peptidyl-prolyl isomerase, and this interaction is required for localization of CK2 to the mitotic spindle. The role of this localization is presumably to position CK2 in the vicinity of specific mitotic substrates associated with the spindle. Along with possible canonical CK2 substrates associated with the mitotic spindle, this also places CK2 in the proximity of numerous Cdk1 substrates, and the phosphorylation status of these Cdk1 sites may influence the ability of CK2 to phosphorylate upstream sites (Figure 5.3). The subtle, multifaceted regulation of a small fraction of cellular CK2α appears to be sufficient for mitotic progression, but in the event of a spindle insult, activation of the SAC leads to an increase in CK2 phosphorylation, recruiting additional CK2 molecules to the mitotic spindle. Collectively, these mechanisms act in concert to allow CK2 to fulfill its mitotic functions.

Figure 5.3

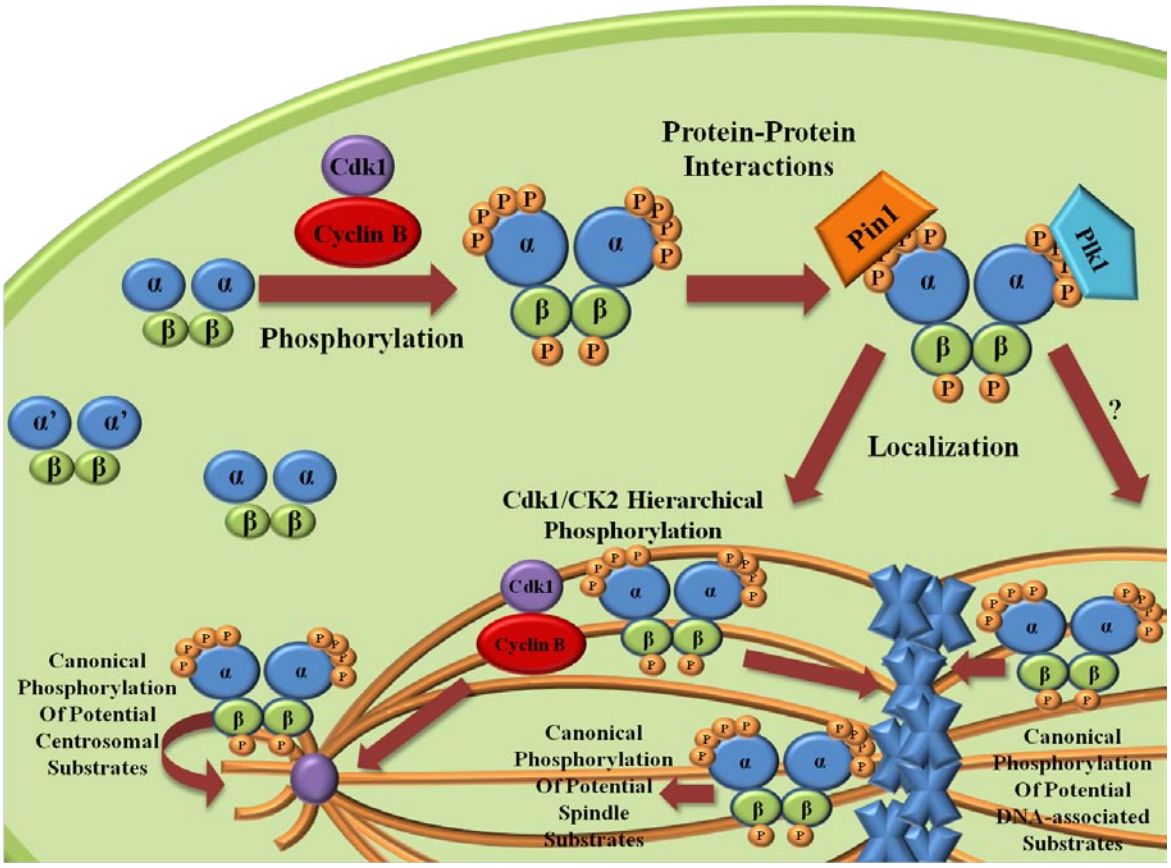

Figure 5.3 Mechanisms of regulating CK2 in mitotic cell division. As Cdk1 is activated at the G2/M transition, it phosphorylates a portion of the cellular CK2, on both the CK2α and CK2β subunits. The CK2α' subunit is not phosphorylated by Cdk1. Phosphorylation of CK2α forms binding sites for both Pin1 and Plk1. The role of the Plk1 interaction is unknown, but the interaction with Pin1 results in the localization of phosphorylated CK2 to the mitotic spindle, where it is presumably targeted towards canonical mitotic spindle substrates. Additionally, it is possible that hierarchical phosphorylation involving Cdk1 and CK2 can contribute to the regulation of CK2 activity for certain mitotic spindle substrates, particularly at the centrosomes and kinetochores.

5.5 Future Directions

5.5.1 CK2 mitotic pathway determination

The work in this thesis has outlined the subtle regulatory forces acting on CK2 during mitosis, but the precise roles CK2 plays in mitotic signalling pathways have yet to be determined. As the results in Chapter 4 indicate that several mitotic proteins are potential substrates for Cdk1/CK2 hierarchical phosphorylation, testing and validation of these substrates may provide a wealth of new insights into the role of CK2 in mitosis. The first step in the validation of these potential substrates would be to generate a peptide array containing the putative consensus sequences from potential mitotic Cdk1/CK2 substrates. As these sequences contain canonical CK2 determinants as well, to compare the relative contributions of the acidic and phosphorylated determinants two peptides could be generated for each sequence, one with serine at the proline-directed site, and another with phosphoserine. The peptide array would be incubated with CK2 and [γ-^{32}P]-ATP to measure the relative phosphorylation of each peptide. The unphosphorylated peptides could also be incubated with Cdk1 and [γ-^{32}P]-ATP to test the specificity of the proline-directed phosphorylation site. Additionally, the unphosphorylated peptides would allow sequential phosphorylation assays, first with Cdk1 and cold ATP, and then with CK2 and [γ-^{32}P]-ATP. By comparing this array to the array incubated with CK2 alone, we could determine the ability of Cdk1 and CK2 to sequentially phosphorylate each peptide. The results of the peptide array experiments would provide an indication of how widespread the effects of hierarchical phosphorylation may be, and more importantly, which substrates may be promising candidates for further study. Promising candidates would next be tested for hierarchical phosphorylation *in vitro* using full length

recombinant proteins, using the same general method as for the peptide arrays. These *in vitro* assays will provide further evidence that these sites represent potential physiological substrates, as a positive result with a peptide substrate, while encouraging, does not reflect the natural environment of the amino acid sequence, which could be buried within a folded protein and therefore inaccessible for phosphorylation. To test the cell cycle specificity of phosphorylation, recombinant substrates could also be incubated with lysates from interphase and mitotic cells and assayed for phosphorylation. Candidate substrates that are phosphorylated *in vitro* could then be investigated in human cells. To test the sites for mitotic phosphorylation, affinity-tagged exogenous proteins would be transfected into cells, and cells would be arrested in mitosis. Upon lysis, the exogenous protein could be immunoprecipitated using the affinity tag, and *in vivo* phosphorylation sites could be identified by mass spectrometry. Phosphorylation-site mutants of candidate substrates could also be expressed in cells to assess the effects of disruption of the sites on mitosis. Mutant forms would include a nonphosphorylatable mutant with alanine residues replacing both sites, a phosphomimetic mutant with aspartic acid residues at both sites, as well as single mutations to both alanine and aspartic acid, which would effectively uncouple the two phosphorylation sites. In particular, a mutant protein with a serine at the CK2 site and an aspartic acid at the Cdk1 site could illuminate the consequences of loss of regulation at these sites, as CK2 could presumably phosphorylate the mutant protein regardless of Cdk1 activation. Since the mitotic peptide match results in Chapter 4 were highly enriched for mitotic spindle components, cells expressing phosphorylation-site mutants of candidate substrates would be assayed for mitotic spindle defects through immunofluorescence and fluorescent microscopy, and for SAC defects

through cell cycle analysis using flow cytometry, as in our study of CK2α phosphorylation-site mutants presented in Chapter 2. These studies would be complemented with rescue experiments, in which the endogenous candidate protein would be depleted using RNA interference (RNAi), and the phosphorylation-site mutants would be assayed for the ability to maintain normal protein function in cells.

In addition to possible mitotic substrates for Cdk1/CK2 hierarchical phosphorylation, identification of canonical mitotic substrates of CK2 would be useful in identifying the precise roles of CK2 during mitosis. To identify these substrates, one approach could be the utilization of CK2 gatekeeper mutants. Chemical genetics studies have demonstrated that when a single hydrophobic residue in the ATP binding pocket of a kinase, termed the 'gatekeeper' residue, is mutated to a smaller amino acid (glycine or alanine), a hydrophobic pocket into the ATP binding site forms (20, 42). Bulky ATP-derivatives that normally cannot fit in the ATP binding pocket are accommodated by the hydrophobic pocket, allowing specific detection of phosphorylation by the kinase of interest in the absence of contaminating signals. Gatekeeper mutants have been generated for several protein kinases, leading to the identification of both known and novel substrates (9). Generation and characterization of CK2 gatekeeper mutants is an area of ongoing investigation in our laboratory, and if a suitable CK2 gatekeeper mutant and ATP derivative are identified, they could be used to identify novel mitotic substrates of CK2, though the incubation of mitotic lysates with the CK2 gatekeeper mutant and ATP-derivative. To isolate and identify phosphorylated peptides, an ATP derivative with a γ-thiophosphate would be used, to facilitate purification of phosphopeptides using iodoacetamide beads as in Figure 5.4. This 'Capture and Release' approach has been used

Figure 5.4

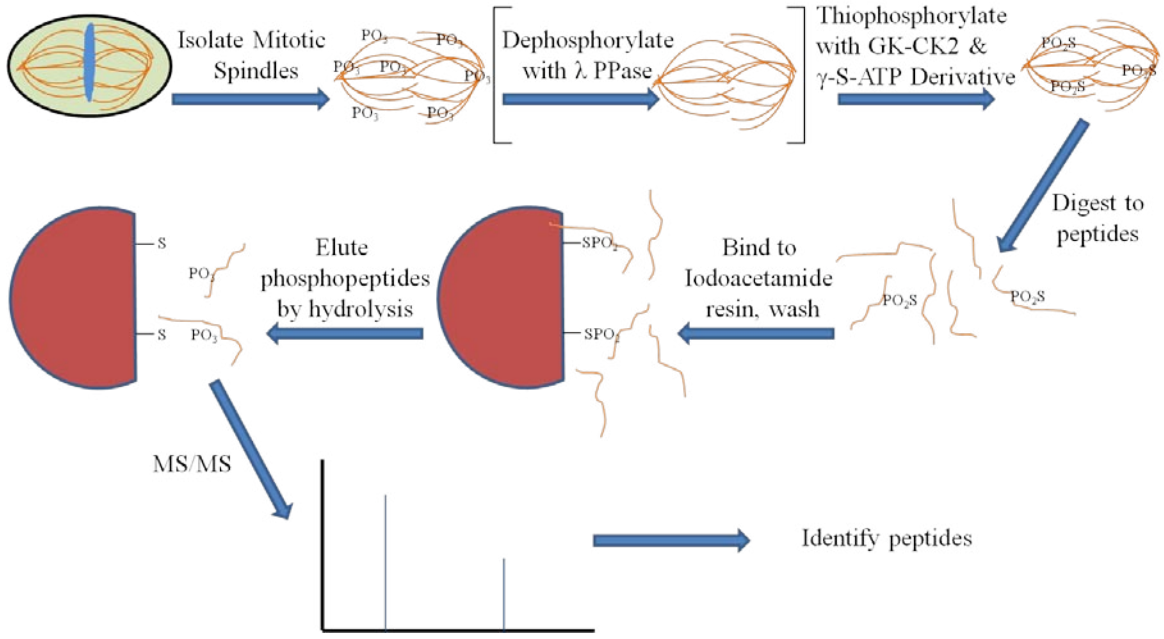

Figure 5.4 A 'capture and release' strategy to identify mitotic spindle substrates of CK2. Mitotic spindles can be isolated from Taxol-stabilized metaphase cells. If desired, the spindles could then be dephosphorylated with lambda phosphatase (λ-PPase) to strip them of *in vivo* phosphorylation sites. The isolated spindles would then be incubated with a CK2 gatekeeper mutant (GK-CK2) and an ATP derivative containing a terminal thiophosphate. After digestion to peptides, thiophoshorylated peptides would bind covalently to iodoacetamide beads, and non-thiophosphorylated peptides would be washed away. Phosphopeptides would then be eluted from the iodoacetamide by hydrolysis, and identified by mass spectrometry.

successfully to identify both previously known and novel mitotic substrates of Cdk1 (3). To specifically identify CK2 substrates on the mitotic spindle, this approach could be combined with the mitotic spindle isolation used in Chapter 3.

CK2 gatekeeper mutants would also allow assessment of the impact of hierarchical signalling using both Cdk1 and CK2 at the same time, which would better mimic the *in vivo* conditions. By incubating the candidate protein with wild-type Cdk1 and [γ-^{32}P]-ATP along with a gatekeeper mutant of CK2 and a [γ-^{35}S]-ATP derivative, we would be able to assess the phosphorylation of a potential substrate for hierarchical phosphorylation and be able to distinguish Cdk1 phosphorylation from CK2 phosphorylation.

Equally important to identifying mitotic substrates of CK2 will be identification of mitotic protein complexes including CK2, particularly those mediated via the CK2α C-terminal phosphorylation sites. Thus far, only two direct interactors of the CK2α have been identified, and identification of higher order complexes could supply great insight on both the roles of CK2 in mitosis as well as additional regulatory forces influencing CK2 activity. A proteomic approach coupling large-scale isolation of complexes with identification of components through high throughput mass spectrometry could be useful in the identification of complexes involving phosphorylated CK2α. To identify direct interactors of the CK2α C-terminus, an *in vitro* GST pulldown approach could be utilized. Indeed, both the known interactions with the CK2α C-terminus, namely Pin1 and the PBD of Plk1, were identified through GST pulldowns (22, 27). To specifically identify proteins interacting with the CK2α C-terminus, truncated C-terminal fragments like those used in Chapter 3 could be used in these studies. To identify phosphospecific

interactions with the CK2α C-terminus, it would be necessary to first phosphorylate CK2α, and this can be readily accomplished through incubation of CK2 with purified active Cdk1 (19, 27). Phosphodependency of interactions could be tested by comparing the interaction profiles of CK2α in the presence or absence of Cdk1.

To potentially identify larger protein complexes that may be associated with CK2 in a mitotic context, affinity purification of CK2 from mitotic cells could be coupled to mass spectrometric analysis. Our laboratory has developed several stable cell lines expressing CK2 with various epitope tags (HA, Myc, TAP) that could be used in this regard. After expressing the exogenous version in cells, the cells would be arrested in mitosis using nocodazole, and CK2 would be purified from the mitotic lysates using antibodies against the attached affinity tag. After digestion to peptides and chromatographic separation, all interacting proteins copurifying with CK2 could be identified. By comparing the composition of the CK2 interactome under different conditions, for example through synchronizing cells at different stages of the cell cycle, the specific complexes important to the various stages of the cell cycle could be identified and studied in detail. To specifically investigate possible protein complexes involving its C-terminal phosphorylation sites, phosphorylation-site mutants of CK2α could be included in the study, and phosphodependent interactions identified by comparing the interaction profiles of the phosphorylation mutants to those of unphosphorylated and phosphorylated CK2α. Comprehensive knowledge of the various complexes involving CK2 will provide insight into the roles of CK2, as well as how it may be regulated in various cellular processes.

5.5.2 CK2β phosphorylation in mitosis

One aspect of CK2 signalling during mitosis was not examined during the course of these studies, namely the mitotic phosphorylation of the regulatory subunit, CK2β. Interestingly, CK2β is also phosphorylated by Cdk1, and therefore its phosphorylation would presumably be temporally regulated similarly to CK2α. However, the function of this phosphorylation event remains unknown. It is possible that the phosphorylated CK2β may act in concert with phosphorylated CK2α to aid in the mitotic localization of CK2 to the mitotic spindle. Additionally, the CK2β phosphorylation site may serve as a binding site for additional mitotic interactions with CK2.

Interestingly, while CK2β is best known as a component of the CK2 holoenzyme, it can associate with other kinases independently of the CK2 catalytic subunits, including c-Mos (5), A-Raf (13), Chk1 (11), and Wee1 (31, 32). It is intriguing to speculate that the mitotic phosphorylation of CK2β may somehow mediate its interactions with these kinases. Indeed, binding to c-Mos, A-Raf, and Chk1 is mediated through the CK2β C-terminus (2), in an area close to its Cdk1 phosphorylation site. Interestingly, interaction of Chk1 with CK2β results in increased Chk1-catalyzed phosphorylation of Cdc25C, an important mitotic phosphatase that is responsible for activation of Cdk1(11). Conversely, the Wee1-CK2β interaction results in inhibition of Wee1 phosphorylation of Cdk1 (31). While CK2β interactions increase Chk1 activity and decrease Wee1 activity, the end result is the same: increased activation of Cdk1, leading to mitotic entry (55). If Cdk1 phosphorylation of CK2β does impact CK2β binding to Wee1 and Chk1, this would represent an additional positive feedback loop, maximizing Cdk1 activation to ensure entry into mitosis.

5.5.3 CK2α/α' chimeric mice

An interesting aspect of this work is that the differential regulation of CK2α and CK2α' during mitosis is one of very few examples of functional divergence between the two catalytic isoforms of CK2. In fact, of the hundreds of known CK2 substrates, very few show a preference for either catalytic subunit, and very few interacting proteins discriminate between the two forms (18). In yeast, deletion of either catalytic isoform does not affect survival, with deletion of both Cka1 and Cka2 required for loss of viability (35). However, while CK2α' knockout mice are viable, albeit with defects in spermatogenesis (50), CK2α knockout mice undergo embryonic lethality at day 10.5, displaying a host of developmental defects, including severe defects in both neural tube and cardiac development (21). This indicates that in mammalian cells, CK2α has at least one additional and crucial function that cannot be performed by CK2α'. The fact that CK2α and CK2α' share over 90% similarity over most of their sequences, yet have completely divergent C-termini (23), suggests that the difference in viability seen in these mice may be due to the presence of the unique CK2α C-terminal domain. While completely different from the shorter C-terminal domain of CK2α', the CK2α C-terminal domain is highly conserved among birds and mammals, including retention of the four mitotic phosphorylation sites (23, 25). In the course of studying the mitotic localization of phosphorylated CK2α, we found that the C-terminus of CK2α when phosphorylated can localize to the mitotic spindle even in the absence of the remainder of the protein. Additionally, while CK2α' showed only minimal spindle localization, a chimeric protein with the CK2α C-terminal domain fused to the conserved portion of CK2α' showed similar levels of spindle localization as CK2α. To determine if the C-terminal domain of

CK2α is the crucial element required for viability, transgenic mice expressing chimeric versions of CK2 (CK2α/α', CK2α'/α) could be generated. By comparing these mice to the CK2α and CK2α' knockout mice, the true impact of the CK2α C-terminus on mammalian life could be elucidated.

5.5.4 CK2 hierarchical phosphorylation in signal transduction

While we set out to determine if Cdk1/CK2 hierarchical phosphorylation can contribute to mitotic signalling, the results of our peptide match search and GO analysis indicate that hierarchical phosphorylation between CK2 and proline-directed kinases can also potentially occur in other pathways, and provide several new avenues for study. In particular, the enrichment of both positive and negative regulators of transcription suggests that hierarchical phosphorylation may significantly affect gene expression. To investigate the effects of hierarchical phosphorylation between CK2 and the various Cdks involved in transcriptional regulation, the approach outlined in section 5.5.1 could be easily adapted.

Additionally, our results on the optimal spacing and number of multiple phosphoserines as phosphodeterminants will be valuable in recognizing possible substrates for non-proline directed hierarchical signalling events involving CK2. Judging by the results of our study, potential substrates for primed phosphorylation would not only require multiple phosphodeterminants, but also the correct spacing of the phosphorylated residues. To search the genome for these types of events, a peptide match search for serine-rich regions could be coupled to searches of various phosphorylation site databases, to identify sequences with known phosphorylation sites in the correct quantity and positioning. Validation of potential substrates could then be carried out as in

section 5.5.1. Given the widespread existence of hyperphosphorylated regions in the human phosphoproteome (37, 51), identification of new substrates regulated in this manner would certainly provide insight into the various roles of CK2 in cellular life.

5.6 Perspectives

Protein phosphorylation regulates every aspect of cellular signalling, yet in many cases the regulatory forces controlling phosphorylation events remain poorly understood. In this thesis, we examined the subtle regulatory mechanisms that enable protein kinase CK2, a constitutively active kinase, to perform regulated functions specific to cell division.

Our study initially focused on four mitotic phosphorylation sites on the unique C-terminus of CK2α. These phosphorylation sites had only been shown in cells arrested in mitosis with the mitotic spindle poison nocodazole, and the generation of phosphospecific antibodies allowed us to examine CK2α phosphorylation in normally progressing mitotic cells for the first time. Our results show that CK2α is phosphorylated in mitosis during prophase and metaphase, consistent with the notion that Cdk1 is the kinase responsible. Expression of CK2α phosphorylation-site mutants in human cells lead to multiple defects in mitosis, including centrosomal amplification, induction of mitotic catastrophe, and abrogation of the SAC. These severe defects demonstrate that CK2 has important roles in mitotic progression, and that proper phosphorylation of CK2α is crucial for the proper execution of these roles.

Since the CK2α phosphorylation sites proved vital for mitotic progression, we hypothesized that phosphorylation of these sites may serve to regulate CK2 activity, and set out to determine the mechanism of this regulation. Since phosphorylation of the CK2α

C-terminal domain does not directly affect CK2 kinase activity, we used the phosphospecific antibodies to examine whether phosphorylation had any effect on CK2 localization. Strikingly, we found that phosphorylated, but not unphosphorylated, CK2α localizes to the mitotic spindle. In addition, we found that localization of phosphorylated CK2α to the mitotic spindle requires the presence of a known phosphodependent interactor of the CK2α C-terminus, the peptidyl prolyl isomerase Pin1. This is the first example of Pin1 acting to localize an interaction partner to the mitotic spindle, and while we were unable to determine if Pin1 actually isomerizes the CK2α phosphorylation sites, we also demonstrated that Pin1 itself localizes to the mitotic spindle, suggesting that Pin1 and CK2α may localize to the mitotic spindle as a complex.

Localization of phosphorylated CK2α to the mitotic spindle may act to target a portion of cellular CK2 towards substrates associated with the mitotic spindle. In order to further examine the regulatory mechanisms acting on CK2 during mitosis, we next chose to examine the possibility that hierarchical phosphorylation between CK2 and Cdk1 may contribute to the regulation of CK2 activity towards certain mitotic substrates. This possibility was intriguing because at these sites, CK2 activity would depend on the activation state of Cdk1, essentially putting CK2 under the same strict regulatory forces. However, our results show that hierarchical phosphorylation between Cdk1 and CK2 is unlikely to occur using strictly primed determinants. Due to this, we next asked whether a downstream Cdk1 site could synergize with weak canonical determinants to form a novel CK2 site. This was indeed the case, and we conducted a genome-wide peptide match search to identify possible candidate substrates. Strikingly, GO analysis revealed that the most highly enriched group of proteins in the peptide match results are components of the

mitotic spindle. This indicates that Cdk1/CK2 hierarchical phosphorylation may have a significant impact on the phosphorylation status of the mitotic spindle. Validation and characterization of these substrates will provide a wealth of information on the roles of CK2 in mitotic regulation.

Collectively, the results in this thesis show that CK2 activity is regulated through phosphorylation, protein-protein interactions, subcellular localization, and hierarchical phosphorylation to ensure proper mitotic progression. This demonstrates that although CK2 is constitutively active, it is indeed subjected to various subtle regulatory mechanisms that allow it to participate in a multitude of cellular signalling pathways. Further work on the regulation and roles of CK2 in various cellular processes, particularly through the use of chemical genetics, proteomics and bioinformatics, will not only expand our understanding of how CK2 acts in these pathways, but also our understanding of the complex integration of cellular signalling.

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Jake Turowec, **Nicole St-Denis**, and David Litchfield (2010). Protein kinase CK2. Submitted for publication as a chapter in **The Encyclopedia of Signaling Molecules**.

Jake Turowec, James Duncan, Ashley French, Laszlo Gyenis, **Nicole St-Denis**, Greg Vilk and David Litchfield (2010). Protein Kinase CK2 is a constitutively-active enzyme that promotes cell survival: Strategies to identify CK2 substrates and manipulate its activity in mammalian cells. **Methods in Enzymology**, In Press.

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