THE CATALYTIC SUBUNITS OF PROTEIN KINASE CK2: EXPRESSION, COVALENT MODIFICATION, AND REGULATORY INTERACTIONS

BY

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Biochemical and genetic studies have demonstrated that protein kinase CK2 (CK2) is one component of the regulatory protein kinase network implicated in cell proliferation and cell cycle progression. Its ubiquitous distribution, lethal effects of gene disruption and high degree of conservation suggest an important role for CK2 in eukaryotic cells, although its exact role in cells remains poorly understood.

CK2 is a protein serine/threonine kinase which is composed of two catalytic subunits (CK2α and/or CK2α') and two regulatory subunits (CK2ß). The CK2α and CK2α' catalytic isozymes are encoded by different genes, are highly conserved between many species of animals and are structurally similar, but differ dramatically at their respective carboxyl terminal domains (CTDs). This observation, coupled with reports that CK2α and CK2α' display different cell cycle dependent intracellular distributions and phosphorylation has led us to hypothesize that functional differences exist between CK2α and CK2α'. In order to test this hypothesis we have: i) examined the expression levels of these isozymes during cell cycle progression, ii) used synthetic peptides and fusion proteins to identify the mitotic sites of phosphorylation within CK2α, and iii) used the yeast two-hybrid system to identify

potential regulatory protein partners.

We have found that, although expression levels of CK2α and CK2α' are relatively similar in cells progressing through the cell cycle, there is a dramatic increase in protein levels of both isozymes after stimulation of cells to enter the cell cycle. Moreover, there are increased levels of CK2ß in mitotically arrested cells. These results suggest that regulation of the protein levels of the subunits of CK2 within cells is important for entry and exit of the cell cycle. We have identified Thr^{344}, Thr^{360}, Ser^{362}, and Ser^{370} as the mitotic sites of phosphorylation on CK2α. Identification of these sites is an important step in defining the role of CKII and its phosphorylation during cell division. We have also identified a novel protein, Phu, which interacts specifically with CK2α, but not with CK2α'.

The work presented in this thesis demonstrates that regulated protein levels of CK2α and CK2α' are important for cell cycle entry, and that differences between CK2α and CK2α' are reflected in isoform specific post-translational modifications and protein:protein interactions.

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There are two very important and special people I want to thank. The first one is my wife and best-friend, Phoebe Bosc. The second person is my supervisor and good friend David Litchfield. Without their guidance and support I would not have made it this far.

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ABBREVIATIONS

AD - Activation domain

AEBSF - 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride

Ala - Alanine

Asp , D - Aspartic Acid

ATP - Adenosine tri-phosphate

BCIP - 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt

BD - Binding domain

BSA - Bovine Serum Albumin

CAK - $p34^{cdc2}$ activating kinase

CDK - cyclin dependent kinase

CK2, CKII or casein kinase II - protein kinase CK2

cDNA - coding DNA

CMV - Cytomegalovirus

CPM - Counts per minute

CTD - Carboxy-Terminal-Domain

C-Terminus - Carboxyl terminus

D-MEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic Acid

DTT - Dithiothreitol

EBV - Epstein-Barr Virus

ECL - Enhanced Chemiluminescence

EDTA - Ethylenediaminetetratacetic acid

EGFP - Enhanced Green Fluorescent Protein

EGTA - Ethylene Glycol-bis (β-aminoethyl Ether)

FBS - Fetal Bovine Serum

GFP - Green Fluorescent Protein

GST - Glutathione S-transferase

HA - YPYDVPDY epitope of influenza virus hemagglutinin

IGF-II - Insulin-like Growth Factor-II

IPTG - Isopropyl-b-D-thiogalactopyranoside

IR - Insulin Receptor

IRS-1 - Insulin Receptor Substrate-1

kDa - kilo Dalton

LB -Luria-Bertani

min. - minute

mL - millilitre

mm - millimetre

mM - millimolar

mRNA - messenger RNA

N-terminus - Amino terminus

NBT - p-nitro blue tetrazolium chloride

ORF - Open Reading Frame

PBS - Phosphate Buffered Saline solution

PH - Pleckstrin Homology

pmol - picomole

PVDF - polyvinylidene difluoride

PMSF - phenylmethylsulfonyl fluoride

PP2A - Protein Phosphatase type-2A

Refs. - References

RNA - Ribonucleic Acid

SDS - sodium dodecyl sulfate

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser, S - Serine

TGN - Trans Golgi Network

Thr, T - Threonine

Tyr - Tyrosine

υL - microlitre

υg - microgram

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cellular growth and proliferation are controlled by various mechanisms. One of these regulatory mechanisms involves the reversible phosphorylation of proteins by a class of enzymes known as protein kinases (1,2,3). This family of enzymes has been implicated in the dynamic regulation of various cellular processes such as, metabolism, gene expression, cytoskeletal architecture, cell adhesion and cell cycle regulation (3). They are critical members of signal transduction pathways which allow cells to respond to extracellular signals (2). Protein kinases represent a structurally diverse group of proteins which differ widely in size, subunit structure, subcellular localization, mechanisms of activation, and substrate specificity. However, all eukaryotic protein kinases are evolutionarily related at a conserved catalytic core (4,5). The non-catalytic regions, which typically display no overall evolutionary relatedness, can be important determinants which guide the subcellular localization and regulation of individual kinases (6). Phosphorylation of seryl, threonyl and tyrosyl residues triggers conformational

changes in protein kinase substrates that alter their biological activities, such as enzymatic activity, localization or binding properties of target proteins. The importance of protein kinases in normal cell function is highlighted by observations that mutations in many protein kinase genes are implicated in a number of human diseases which include diabetes and cancer. For this reason, it is imperative that we study the function and regulation of these proteins in order to devise strategies aimed at regulating their activities in order to control any adverse effects in human health caused by dysregulation of kinases.

One component of the protein serine/threonine kinase network implicated in regulation of cell proliferation and cell cycle progression is protein kinase CK2 (CK2). The exact nature of the participation of CK2 in these cellular events is not completely understood. It is therefore the goal of the research presented in this thesis to contribute to a better understanding of the role and regulation of CK2 in mammalian cells.

GENERAL FEATURES OF CK2 -

Protein Kinase CK2 (CK2) is a protein serine/threonine kinase involved in a wide variety of cellular processes of all eukaryotes examined thus far (7,8,9,10,11, and 12). It has been suggested that CK2 is a constitutively active

housekeeping enzyme (10,13,14)

CK2 is unique among the other kinases in that it can utilize either ATP (approx. Km 10 uM) or GTP (approx. Km 20 uM) as substrates (8, 15). In the presence of Mg^{2+}, ATP is the more efficient substrate but in the presence of Mn^{2+}, the situation is reversed (10). CK2 is also known as casein kinase II because of its ability to phosphorylate serine/threonine residues of casein *in vitro*. The name "casein kinase II" has been changed in favor of protein kinase CK2 because of the possible misleading connotation that casein is a physiological substrate of CK2 (10). Its designation arises from observations that CK2 elutes from a DEAE-cellulose chromatographic column as the second casein kinase (16). In fact, CK2 elutes from DEAE-cellulose with monovalent cation concentrations between 0.15 and 0.25 M (8). Furthermore, CK2 can bind readily to phosphocellulose and can be removed at a monovalent ion concentration of 0.7M (8). These biochemical properties allow for effective purification of CK2. Although exceptions have been reported in some organisms, the CK2 holoenzyme is typically purified from eukaryotes as an active tetrameric holoenzyme of molecular mass 130,000 Da (7). The CK2 holoenzyme is comprised of two catalytic subunits (CK2α and/or CK2α') and two regulatory subunits (CK2β) (8,16,17,18).

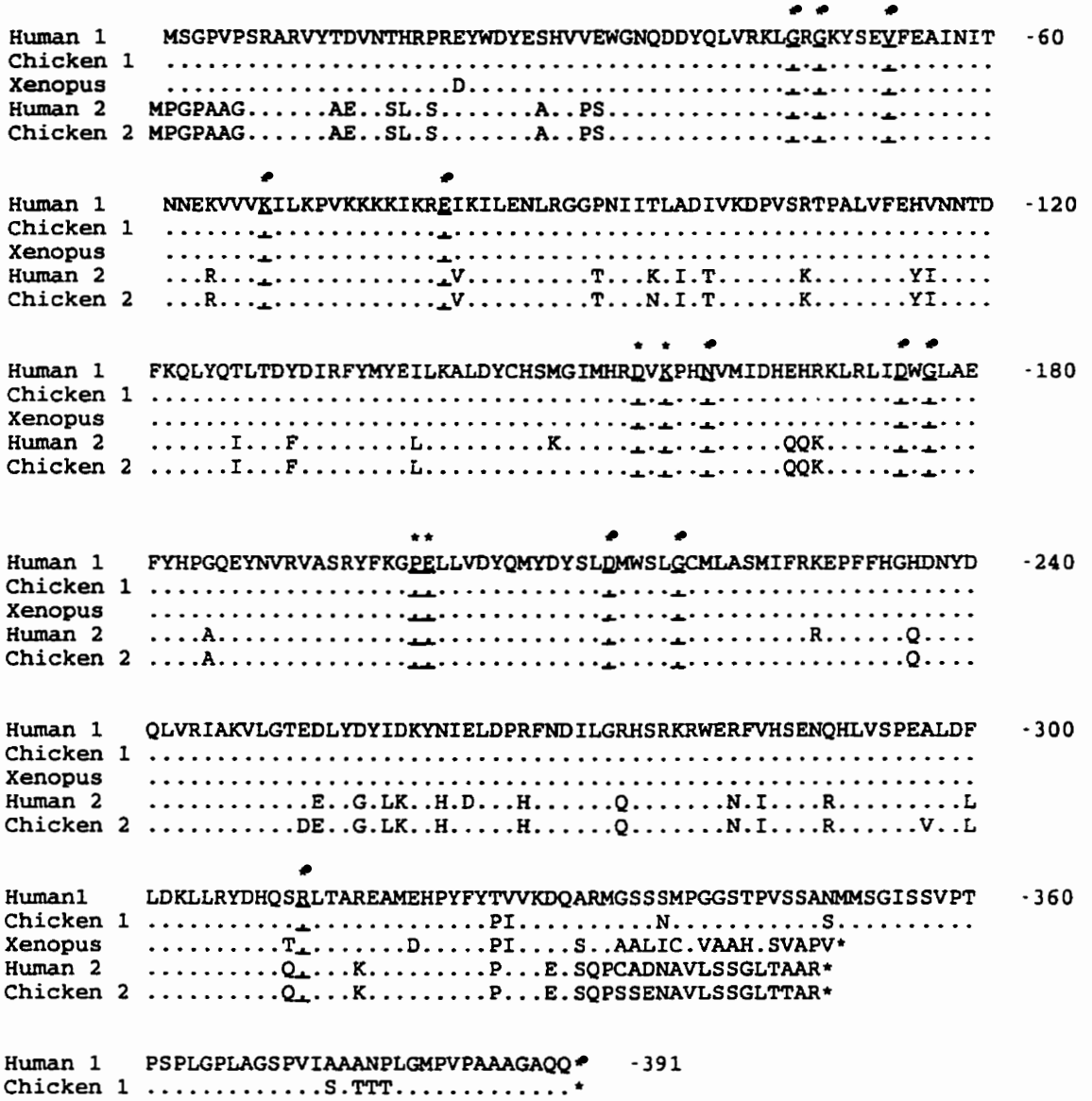

Fig. 1.1 **Alignment of deduced amino acid sequences of CK2α and CK2α' subunits from vertebrates.** The following sequences are shown: human CK2α (human 1), human CK2α'(human 2), chicken CK2α (chicken 1), chicken CK2α'(chicken 2), and *Xenopus laevis* CK2α. Numbering of amino acids corresponds to the numbering of human CK2α. Residues that are conserved in all members of the protein serine/threonine kinase family are underlined and indicated with an asterisk. (19)

CATALYTIC SUBUNITS OF CK2 -

The CK2α and CK2α' subunits are catalytically active by themselves and have an Mr between 41-44 kDa (CK2α) or 37-42 kDa (CK2α') (7,8,10,11,12,20,21,22,23,24). Human CK2α and CK2α' are the products of separate genes (24,25) which are located on chromosomes 20p13 and 16p13.3-13.2 for CK2α and CK2α' respectively (26,27).

In mammals and birds, the CK2α and CK2α' subunits exhibit a very high degree of identity (see Fig.1.1)(19). The 330 N-terminal amino acid sequences are greater than %90 identical between CK2α and CK2α', and it is within these N-terminal regions that CK2α and CK2α' contain the protein kinase superfamily hallmark regions I to XI (28). Region I is involved in the binding of ATP or GTP. In CK2α, this region contains a Tyrosine residue (Tyr^{50}) in a position typically found in the cdk family of kinases (10). A major difference between CK2 and other kinases is found within a stretch of amino acid residues located between regions II and III. In most kinases, this area is comprised of acidic residues, however in CK2α, this area contains many basic residues (K^{74}-K-K-K-I-K-R^{80}). The basic nature of this region is, believed to direct interactions with acidic regions of CK2 substrates, shown to be important in regulating CK2 activity with polyanionic compounds, and also contains elements consistent with a nuclear localization signal (10,29).

Region VI contains an unusual His^{148}-X_5-His^{154}-X_5-His^{160}-X_5-His^{166} arrangement flanked on either side by a Lys^{142} and an Arg^{172}. The function of this intriguing arrangement is currently unknown, but it has been speculated to play a role in substrate recognition (10).

The most striking difference between CK2α and CK2α' occurs at their Carboxyl-Terminal-Domains (CTDs).

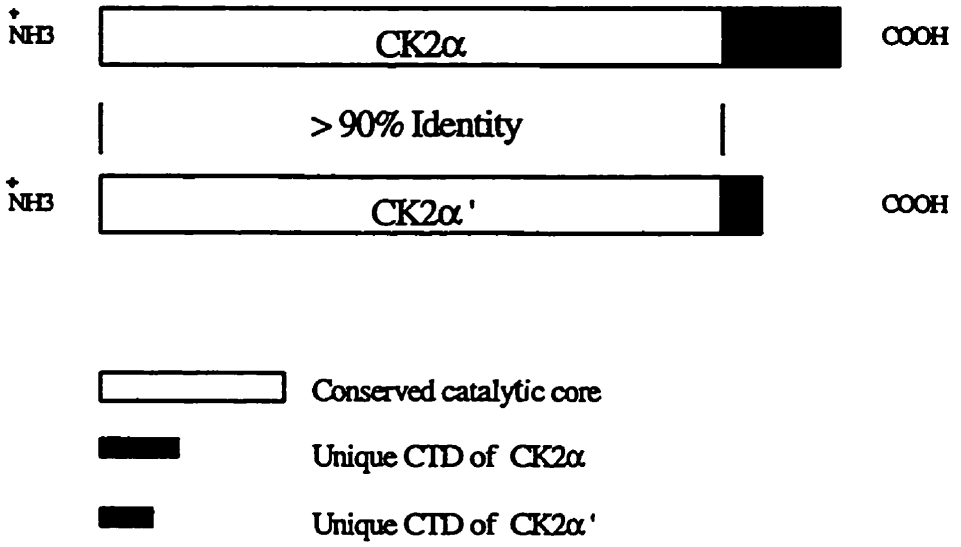

Fig. 1.2 **Schematic diagram of the CK2α and CK2α' catalytic subunits of human protein kinase CK2.** The unique 61 amino acid and 20 amino acid Carboxy-Terminal-Domains (CTDs) of CK2α and CK2α' respectively, are highlighted.

The unrelatedness and uniqueness of the CTDs of CK2α and CK2α' suggest functional differences between the CK2α and CK2α' isoforms may exist in cells (19). In fact, I have observed that the CTD of CK2α, but not CK2α', possesses a "P-X-X-P" motif encompassing amino acid residues 363-366. This type of motif has been demonstrated to be important for mediating the interaction with SH3 domains of certain proteins (30,31,32). The presence of a "P-X-X-P" motif within the unique CTD of CK2α suggests that CK2α may have the ability to engage in unique protein:protein interactions which may not be available to CK2α'.

Attempts have been made to determine whether functional differences exist between CK2α and CK2α'. An *in vitro* study using purified recombinant human CK2α and CK2α' subunits failed to identify any significant catalytic differences between the isoforms (33). The findings of this comprehensive study are not surprising, since the catalytic domains of CK2α and CK2α' are closely related. The only differences observed between CK2α and CK2α' were that, CK2α has an isoelectric point at pH 7.3, while CK2α' at pH 8.6, and that, under certain conditions, CK2α', but not CK2α, undergoes autophosphorylation, of which the significance is unclear (33).

Major differences between CK2α and CK2α' in cells were reported (34,35). Yu et al.,(1991) described cell cycle dependent changes in the subcellular localization of both

CK2α and CK2α'. Those observations are further discussed in section: "Localization of CK2" of this introduction/thesis. Meanwhile, Litchfield et al. (1992), observed that CK2α, but not CK2α', is phosphorylated during mitosis. Phosphopeptide mapping studies demonstrated that the same peptides which were phosphorylated on CK2α in mitotic cells were also phosphorylated *in vitro* by the $p34^{cdc2}$ kinase, suggesting that CK2α is a physiological substrate of $p34^{cdc2}$. Altogether, these observations imply that CK2α and CK2α' may be subject to different modes of regulation, and that they may possess independent functions during cell cycle progression.

REGULATORY SUBUNIT OF CK2 -

CK2β by itself has no catalytic activity, but it can upregulate and stabilize the activity of CK2α and CK2α' (33,36, 37,38,39,40,41,42). In general,CK2β can stimulate the catalytic activity of CK2α or CK2α' 5- to 10-fold towards many substrates (10),however, one notable exception is the phosphorylation of calmodulin which is decreased in the presence of CK2ß (33, 43).

The gene encoding human CK2β is located at 6p21.1 (44), and codes for a protein of 26 kDa (8). Interestingly,CK2β does not share any identity with the catalytic or regulatory

subunits of other protein kinases (19).CK2β displays an extra-ordinary level of conservation between different species, in fact, in vertebrates, there is only 1 amino acid difference between CK2β of *Xenopus laevis*, mouse, chicken and human (19). Furthermore, there are only 26 amino acid differences between *Drosophila melanogaster* and *Homo sapiens* (10). Some interesting features of CK2β include the presence of a possible zinc finger and a putative "cyclin destruction box" (10).

EXPRESSION PATTERNS OF CK2 SUBUNITS -

Northern blot analysis of CK2 subunits have shown that CK2α and CK2α' have dramatically different expression levels in various adult chicken tissues (45). For example, spleen and heart tissues exhibited high levels of CK2α, but very little CK2α'. Conversely, the CK2α' signal was highest in liver, brain and ovary tissue, while CK2α levels were much lower. These results suggest that CK2α and CK2α' may have specialized functions in different tissues. The CK2β mRNA levels were easily detected in all tissues examined (45).

Western blot analyses of the CK2 subunit protein levels

in various adult chicken tissues did not vary as much as the Northern blot results (46). Krek et al., (1992), reported that CK2α, CK2α', and CK2β proteins are most abundant in brain, but scarce in liver (46). CK2α protein levels were similar in all other tissues examined, except for muscle, where the levels were comparatively quite low (46). Protein levels of CK2α' and CK2β were very low in all tissues examined, aside from brain (46). These same authors also reported that the relative protein amounts of CK2α, CK2α', and CK2β were quite comparable (46), unlike the levels of CK2 mRNAs detected by Maridor et al., (1991). This led Krek et al., (1992) to speculate that translational and/or post translational mechanisms may function to control the protein levels of CK2 subunits (46).

CK2 HOLOENZYME COMPOSITION AND FORMATION -

CK2 composition - The CK2α, CK2α' and CK2β subunits can associate to form the following heterotetrameric or homotetrameric structures in cells: $CK2\alpha_2/CK2\beta_2$, $CK2\alpha/CK2\alpha'/CK2\beta_2$, or $CK2\alpha'_2/CK2\beta_2$ (8,24 47,48). Using the yeast two-hybrid system (48), various groups were able to demonstrate that CK2β is able to interact with itself, CK2α,

and CK2α'. It was also shown that neither CK2α nor CK2α' could homodimerize nor heterodimerize (48,50,51).

Recently, many laboratories have endeavoured to identify the domains of interaction between CK2α and CK2β. Results obtained by using the yeast two hybrid system, together with experiments using synthetic peptides, have been informative, although inconsistent in some instances. In yeast two-hybrid system experiments, Kusk et al., (1995), reported that amino acid residues 20-145 of CK2β are involved in CK2β homodimerization, while CK2β residues 152-200 are important for CK2α and CK2β hetero-dimerization (50). Similar results were obtained by Litchfield et al., (1996), who demonstrated that CK2β residues 133-215 are important for CK2α and CK2β hetero-dimerization (52). Boldyreff et al., (1996) identified residues 156-165 of CK2β as being important for homodimerization of CK2β subunits (51). Identification of the residues within CK2α that are important for CK2α and CK2β dimerization has proven to be difficult. For instance, in one laboratory workers essentially observed that only full length CK2α was able to interact successfully with CK2β (52), while in another laboratory, it was reported that deletion of either the 21 N-terminal or the 41 C-terminal

residues of CK2α, which should leave the kinase domain intact, did not affect the ability of CK2α and CK2β to heterodimerize (50).

Complimentary *in vitro* interaction studies using synthetic peptides have proven useful. Synthetic peptides encoding CK2β residues 155-215 (53) or 155-181 (54) can stimulate CK2α activity. Furthermore, Krehan et al., (1998), demonstrated that residues 162-175 of CK2β are involved in tight association with CK2α (54). Marin et al., (1997), showed that CK2β residues 155-170 are important for interaction with CK2α, and that CK2β peptide 155-215 displayed the ability to self associate (55). Of note, is the observation that a recombinant deletion mutant lacking residues 171-215, failed to form tetrameric complexes with CK2α (56).

Altogether, these results indicate that the C-terminus of CK2β is important for homodimerization as well as hetero-dimerization with CK2α, and presumably with CK2α'. Furthermore, it appears that the CTD of CK2α is not essential for interaction with CK2β.

CK2 holoenzyme formation in cells - The exact nature and

sequence of events in the formation of the CK2 holoenzyme in cells remains to be elucidated. However, experiments by various laboratories have shed some light into this area.

Biosynthetic labeling studies using transformed cell lines have indicated that CK2β is synthesized in excess over CK2α, and that free CK2β has a significantly shorter half-life than CK2β incorporated into the CK2 holoenzyme (57). Immunoprecipitation analyses from HeLa cell extracts demonstrated that the majority of CK2 is assembled in the nucleus from free CK2 subunits (47). Furthermore, these authors also found that the $CK2\alpha'_2/CK2\beta_2$ tetrameric structure forms more quickly than either $CK2\alpha_2/CK2\beta_2$ or $CK2\alpha/CK2\alpha'/CK2\beta_2$ (47).

It has been proposed that CK2β dimer formation is a prerequisite for the subsequent association of the CK2α or CK2α' subunits to form a CK2 tetrameric holoenzyme. (51, Graham and Litchfield, unpublished observation). Furthermore, expression of epitope-tagged kinase-dead CK2α or CK2α' subunits in Cos 7 cells demonstrated that kinase activity is not essential for the formation of complexes between CK2 subunits (58).

An interesting study on the "Quaternary Structure of Casein Kinase II" was reported by Valero et al., (1995). In

this report, the authors identified four major different oligomeric forms of CK2, whose formation was dependent on the ionic strength of the aqueous solution (59). In its most active form, CK2 has a ring-like structure which can dissociate into less-active protomers. These results suggest that the structure of CK2 is important for its activity, and that CK2 activity may be regulated in cells by regulatory ligands which may affect CK2 structure.

SUBSTRATE SPECIFICITY OF CK2 -

Using synthetic peptides and purified proteins as *in vitro* substrates for purified CK2, it was determined that CK2 preferentially phosphorylates serine and threonine residues of substrates adjacent to a cluster of acidic amino acids. (7,8,60). The presence of acidic residues located C-terminal to the phosphoacceptor amino acid is crucial. Acidic residues on the N-terminal also strongly influence the phosphorylation efficiency (7,8,60). The presence of an acidic residue at the n+3 position (e.g. Glu, Asp, or even phosphoserine or phosphotyrosine residues), is critical for the phosphorylation of a substrate by CK2. Furthermore, CK2 preferentially phosphorylates serine over threonine as shown by the RRREEESEEE peptide, which has a V_{max}/K_m ratio 10 fold higher than the RRREEETEEE peptide (61). This information has led to the prediction of a minimal CK2 consensus

sequence for substrate recognition and phosphorylation which is typically represented as: "S/T-X-X-Acidic residues", where the "X" residues appear to be any amino acid, except for a Proline residue at n+1 position which can negatively affect CK2 recognition (7,8,62). It is important to note that not all CK2 substrates are phosphorylated at a CK2 consensus sequence (19).

SUBSTRATES / PROTEIN PARTNERS CK2 -

The aim of this section is to highlight some of the most notable substrates and partners of CK2.

There are more than 100 reported CK2 substrates (see Table 1.1), which are proteins implicated in a wide variety of cellular functions (7,8,9,10,11,12,15,19). Typically, proteins are deemed CK2 substrates because of the ability of CK2 to phosphorylate sites on these proteins which are known to be phosphorylated in cells. Phosphorylation of many of these substrates by CK2 has been shown to affect their biological activity, while in other instances, the effects of CK2 phosphorylation are not understood.

Table 1.1 - **List of notable substrates of CK2** (refs)

Transcription factors -

ATF-1 (63)
c-Jun (64)
c-Myb (65)
c-Myc (66)
Cut (67)
E47 (68)
Max (69)
MDM2 (70)
mUBF (71)
p53 tumor suppressor (72)
Tal 1 (73)

Signal Transduction Proteins -

Calmodulin(74)
Caveolin (75)
Endoplasmin (76)
FK506 (77)
Glycogen Synthase (78)
IkBa (79)
IkBb (80)
IGF-II receptor(81)
Insulin receptor (82)
IRS-1 (83)
Nopp140 (84)
PKA R-II subunit (85)
PKC(86)
PP2A (87)
p34cdc2(88)
Serum response factor (89)

Cytoskeleton and structural proteins -

Caldesmon (90)
Gap43 (Neuromodulin)(91)
MAP-1B(92)
Myosin light chain (93)
Myosin heavy chain (94)
p65 (Synaptotagmin)(95)
Spectrin (96)
Tau (97)

Intracellular Trafficking -

Calnexin (98)
Dynamin (99)
Dynein (100)
Furin (101)
Mannose-6-phosphate receptor (102)

Nucleic Acid Synthesis-

DNA ligase(103)
DNA topoisomerase I (104)
DNA topoisomerase II(105)
RNA polymerase I (106)
RNA polymerase II (107)
RNA polymerase III(108)

Other proteins -

eIF-2(109a and 109b)
eIF-3& 4 (110)
eIF-5 (111)
GEF (112)
Hsp90 (113)
Nucleolin (114)
Nucleoplasmin (115)
Osteopontin (116)
Proteasome complex(30 kDa)(117)
SV40 Large T (118)

Table 1.2 - **List of CK2 "Partners"**

Associated Proteins / Cell Structures(*)	CK2 Subunits Involved	Refs.
A-Raf	CK2β	(119,120)
ATF-1	CK2α / CK2α'	(121,122)
DNA Topoisomerase II	CK2β	(123)
CD5	CK2β	(124)
HSP-90	CK2α	(125)
Mos	CK2β	(126)
Nopp140	CK2β	(127)
Nucleolin	CK2α / CK2α'	(128)
$p21^{WAF1/CIP1}$	CK2β	(129)
p53 tumor supressor	CK2β	(130,131,132)
PP2A	CK2α	(87)
*Caveolae	?	(75)
*Mitotic spindle	All	(34,46)
*Nuclear Matrix	?	(133)
*Plasma Membrane	CK2β	(134)
*Proteaosome(30-kDa)	?	(117)

? Not known

NUCLEAR PROTEINS -

The first group of CK2 substrates / associated proteins to be discussed are the nucleic acid associated proteins. Included among this group are the transcription factors: ATF-1, c-Myb, c-Jun, Serum Response Factor, and the tumor supressor p53. Also of note are: DNA Topoisomerase II, FK506-binding protein (FKBP25), Nucleolin, Nopp140, and UBF.

CK2 and ATF-1 - ATF-1 is a member of the ATF family of transcription factors which has been co-purified with CK2 using DNA-affinity latex beads (121). Recently, Yamaguchi et al., (1998) demonstrated that CK2α or CK2α' can bind to the basic leucine zipper (bZIP) DNA-binding domain of ATF-1 as well as that of other transcription factors such c-Jun, c-Fos, CREB and CREB-BP1 (122). The basic region of the ATF1 bZIP motif was found to be critical for the interaction with CK2α, and that the leucine zipper may modulate binding. Moreover, interaction between CK2α and the bZIP motif of ATF1 appears necessary for the phosphorylation of ATF1 at its N-terminal Ser^{36}. These results led Yamaguchi et al., (1998) to speculate that ATF1 recruits CK2 to promoter DNA regions, thus allowing CK2 to be a participant in transcriptional regulation by phosphorylating nearby proteins (122).

CK2 and c-Myb / c-Jun / SRF - Phosphorylation of c-Myb and c-Jun by CK2 appears to negatively regulate their DNA binding activities (64, 65). C-Myb is phosphorylated at N-terminal Serine residues (Ser^{11} and Ser^{12}) by CK2, while the c-Jun oncoprotein is phosphorylated at C-terminal Threonine and Serine residues (Thr^{231} and Ser^{249}) (64,65). Interestingly, the target residues for CK2 phosphorylation are not present in the majority of oncogenically-activated forms of Myb (65). Phosphorylation of c-Jun, which is one component of the AP-1 transcription factor able to bind to the TRE (TPA response element) DNA sequence, decreased its ability to bind to the TRE sequence (64). Furthermore, micro-injection of synthetic peptide CK2 inhibitors in cells caused an induction of AP-1 activity (64). This effect could be overcome by micro-injection of purified CK2 (19).

In contrast to the inhibitory role of phosphorylation of c-myb and c-Jun, is the phosphorylation of the Serum Response Factor (SRF). Phosphorylation of SRF by CK2 appears to increases its rate of DNA association by altering the conformation of SRF's DNA binding domain (89, 135). *In vitro* studies by Marais et al., (1992) demonstrated that CK2 phosphorylation acted to potentiate SRF-DNA exchange rates but did not affect the binding affinity between SRF and DNA (136). Gauthier-Rouvière et al., (1991), showed there was a marked increase in SRF phosphorylation in cells, following micro-injection of CK2 in quiescent cells (137).

CK2 and the tumor supressor p53 - The association between CK2 and the tumor supressor p53 is quite intriguing. P53 and CK2 were observed to copurify from SV40-transformed NIH-3T3 cells, and CK2 activity was detected in p53 immunoprecipitates (72). Similarly, Filhol et al., (1992), were able to co-immunoprecipitate ectoptically overexpressed CK2 and p53 from infected Sf9 insect cells (130). Moreover, using purified recombinant p53, CK2α, and CK2β proteins, these same authors formed a stable p53-CK2 complex *in vitro* (130). Interestingly, the CK2 holoenzyme, but not CK2α itself, could readily phosphorylate p53, suggesting that CK2β, but not CK2α, is necessary for the formation of the CK2-p53 complex (130,131). Further studies demonstrated that the formation of the p53-CK2 complex is mediated through the association of CK2β with the C-terminal region of p53 (130, 131). Specifically, p53 amino acid residues 287-340, and CK2β amino acids 72-149 are crucial for the interaction between CK2β and p53 *in vitro* (132).

p53 is phosphorylated by CK2 at its penultimate amino acid (Ser^{392} in humans), and is an example of a CK2 phosphorylation site which does not conform to a CK2 consensus sequence (138). The role of p53 phosphorylation by CK2 is not clearly understood, although it appears necessary for the suppressor function of p53, since replacing the

target Serine residue with a non-phosphorylatable Alanine residue abolishes the ability of p53 to suppress cell proliferation (139). The mechanism leading to this observation is unknown, but it is believed that phosphorylation of p53 activates its DNA binding ability (140). This led to the speculation that phosphorylation of p53 by CK2, may regulate the transcriptional activation properties of p53. However, experiments using wild type and p53 phosphorylation mutants failed to show any differences in the ability of p53 to bind DNA or activate transcription (138, 141, 142,143). Interestingly, Hall et al., (1996), observed that the non-phosphorylatable alanine p53 mutant displayed a decreased ability to repress expression from a c-fos promoter, suggesting that phosphorylation of p53 by CK2 may be important in regulating p53-dependent repression (143). In contrast, Hao et al., (1996) demonstrated that although mutated p53 did not behave any differently than wild type p53 in proliferating cells in G_1-arrested cells, only the p53 mutant (Serine -> Glutamate) retained the ability to bind DNA and possess transactivation function (144).

Overall, the role of the phosphorylation of p53 by CK2 is unclear. Although it appears to be important in DNA binding of p53, Wagner et al., (1998), recently reported that *in vitro* phosphorylation of p53 by purified CK2 resulted in a significant reduced binding affinity between p53 and $p34^{cdc2}$

(145). Similar results were obtained when the CK2 phosphorylation site of p53 was replaced with an aspartic acid residue (145).

CK2 and DNA topoisomerase II - DNA topoisomerase II is one of the best characterized of the CK2 substrates and protein partners. Topoisomerase II is phosphorylated in cells at multiple sites, within its carboxy-terminus, which are also phosphorylated *in vitro* by purified CK2. (105,146,147).

The utilization of *Saccharomyces cerevisiae* conditional mutants has been of prime importance in studying the phosphorylation of topoisomerase II by CK2. These mutants behave normally at a permissive temperature, however CK2 activity is dramatically reduced upon incubation of these cells at a restrictive temperature (12). Incubation of one of these conditional CK2 mutants (cka2-8^{ts}) at the restrictive temperature, resulted in a large decrease in topoisomerase II phosphorylation, as compared to wild type cells, or cells grown at the permissive temperature (147). Furthermore, it was demonstrated that topoisomerase II is most highly phosphorylated by CK2 during metaphase in the *Saccharomyces cerevisiae* mutants (146,147).

Interaction between topoisomerase II and CK2 has been shown in experiments in which topoisomerase II and CK2 co-immunoprecipitate and biochemically co-purify from

Saccharomyces cerevisiae cell extracts, while purified recombinant CK2 and topoisomerase II form a stable and active CK2/topoisomerase II complex *in vitro* (123). Bojanowski et al., (1993), were able to determine that CK2β is necessary for the formation of the complex, suggesting that CK2 and topoisomerase II interact in cells through the CK2β subunit of CK2 (123).

The role of the CK2 association and phosphorylation of topoisomerase II is not clear. Ackerman et al., (1985), as well as Cardenas et al., (1993), reported that *in vitro* phosphorylation of topoisomerase II by CK2 enhanced its ATP-dependent decatenation activity (105,148). However, Kimura et al., (1996) found no enhancement of topoisomerase II activity upon phosphorylation by CK2 *in vitro* (149). Dang et al., (1994) observed that phosphorylation of topoisomerase II by CK2 enhanced the stability of the topoisomerase II/DNA complex, but this observed effect was limited to linear or relaxed circular DNA (150). Interestingly, there is a recent report showing that bufalin treatment of human leukemia (U937) cells resulted in apoptosis of these cells by stimulating nuclear transport of CK2 into the nucleus causing premature phosphorylation and activation of topoisomerase II (151).

CK2 and Nopp140 - Nopp140 is a nucleolar protein which was

isolated as a nuclear localization sequence (NLS) binding protein (152). Nopp140 is believed to function as a chaperone for import and/or export to/from the nucleolus (84,153). The observations that Nopp140 is highly phosphorylated in cells, presumably by CK2, and that phosphorylation of Nopp140 by CK2 enabled it to bind to a NLS containing peptide prompted Meier and Blobel,(1992), to propose that phosphorylation of Nopp140 by CK2 may regulate the shuttling of Nopp140 between the cytoplasm and the nucleolus (84). In recent studies, Nopp140 was observed to co-immunoprecipitate with CK2α, and to interact directly with CK2β *in vitro* (127), suggesting an *in vivo* association between Nopp140 and CK2 through the CK2β subunit. Formation of a Nopp140/CK2 complex may be important in regulating the localization of CK2 in the cytoplasm and nucleus.

CK2 and nucleolin - Nucleolin is a protein which has been implicated in rDNA transcription, preribosome assembly, and nucleolar chromatin organization. Experiments aimed at determining whether CK2 could phosphorylate FKBP25 (FK506-binding protein), resulted in the observation that GST-FKBP25 interacts with a protein complex comprised of CK2 and nucleolin in cell extracts (154). It was later shown that CK2α or CK2α' can interact directly with nucleolin *in vitro*, and that nucleolin co-immunoprecipitates with CK2

(128).

In confluent cells, where transcription of rRNA is reduced to 5% of the transcription level observed in growing cells, nucleolin is not phosphorylated (155). In actively growing cells, nucleolin is phosphorylated on many serine residues by CK2 during interphase (155), or by $p34^{cdc2}$ on many threonine residues during mitosis (156,157). Phosphorylation by CK2 appears to upregulate rDNA transcription, while phosphorylation by $p34^{cdc2}$ is correlated to mitotic reorganization of nucleolar chromatin (156).

CK2 and UBF - Upstream Binding Factor (UBF) is a nucleolar transcription factor for RNA polymerase I which is phosphorylated by CK2 on serine residues located within its C-terminus (71,158,159). Phosphorylation of UBF by CK2 at its C-terminus increases its transactivation activity, but not DNA binding activity, which results in transcription of ribosomal RNA genes (71).

Considering that CK2 has the ability to activate UBF through phosphorylation, and that ribosomal gene transcription and phosphorylation of the UBF correlate strongly with the growth rate of cells (158), one may infer an interesting correlation between rRNA synthesis, CK2 activity, and cell proliferation.

Collectively, phosphorylation of nucleolin, as well as UBF, support an important function for CK2 in rRNA synthesis

during cell proliferation.

Overall, CK2 phosphorylation of nuclear proteins, such as transcription factors, is important in regulating their functions in cellular proliferation. Furthermore, the observation that topoisomerase II is highly phosphorylated by CK2 during mitosis further implies a role for CK2 in mitosis.

SIGNAL TRANSDUCTION PROTEINS -

The second group of CK2 substrates / protein partners is made up of proteins involved in signal transduction. This group includes proteins such as: $p34^{cdc2}$, Insulin receptor (IR), Insulin receptor substrate-1 (IRS-1), calmodulin, protein phosphatase type-2A (PP2A), and IkB-α.

CK2 and p34^{cdc2} - $P34^{cdc2}$ is a protein serine/threonine kinase that plays a key role in the control of the eukaryotic cell division control (160,161,162).

A strong association between CK2 and $p34^{cdc2}$ is observed by their mutual ability to phosphorylate each other (35,88), as well as some of the same substrates such as: p53, caldesmon, and nucleolin (156,157,163,164). Russo et al., (1992), demonstrated the $p34^{cdc2}$ is phosphorylated on Ser^{39} by CK2 during the G_1 stage of the cell cycle (88). These authors speculated that the phosphorylation of $p34^{cdc2}$ by CK2

would make $p34^{cdc2}$ unable to interact with a cyclin partner, thus maintaining $p34^{cdc2}$ in a inactive monomeric form (88). However, no other reports addressing this phenomenon have been published. Meanwhile, Litchfield et al., (1992), observed that $p34^{cdc2}$ phosphorylates both the CK2α and CK2β subunits of CK2 in mitotic cells (35). Phosphorylation of either CK2α or CK2β doesn't appear to affect CK2 activity, or the ability to form a CK2 holoenzyme (35, 165). Although the physiological relevance of the mitotic phosphorylation of CK2α and CK2β is currently unknown, Litchfield et al., (personal communication), have observed that mitotic phosphorylation of CK2α enables it to interact with the petidyl-prolyl isomerase, Pin1. Pin1 is a protein which can bind, and presumably regulate, mitosis-specific phosphoproteins (166,167).

The ability of CK2 and $p34^{cdc2}$ to phosphorylate each other in a cell cycle dependent manner, along with the "sharing" of certain substrates provides evidence of an intimate association between the two kinases.

CK2 and IRS-1 / calmodulin - A myriad of serine/threonine protein kinases, including CK2, have been reported to be activated in response to insulin in certain cell lines (168,169,170,171). However, CK2 activation by insulin is controversial and may be cell type dependent (19,172).

A role for CK2 in insulin signal transduction is supported by the observations that both IRS-1 and calmodulin are substrates of CK2. IRS-1 is phosphorylated on Thr^{502} by CK2 *in vitro* (83), while calmodulin is most likely phosphorylated in cells at residues Thr^{79}, Ser^{81}, and Ser^{101}, and Thr^{117} by CK2 (74,173,174). IRS-1, which functions as an intracellular regulatory protein that transduces the insulin signal to a variety of proteins through rapid phosphorylation by the insulin receptor tyrosine kinase in an insulin-dependent manner, is also phosphorylated on serine/threonine residues prior to insulin stimulation. It is speculated that serine/threonine phosphorylation of IRS-1 modulates IRS-1 activity by attenuating or desensitizing the effects of basal and insulin stimulated tyrosine phosphorylation of IRS-1 (175,176). The interesting link between IRS-1 and calmodulin is that both are phosphorylated by the insulin receptor tyrosine kinase. Insulin is reported to stimulate the phosphorylation of calmodulin on serine, threonine and tyrosine residues (173). Moreover, Sacks et al., (1992), demonstrated that pre-phosphorylation of calmodulin by CK2 significantly enhanced the ability of the insulin receptor to phosphorylate calmodulin (173).

Altogether, these observations suggest that CK2 phosphorylation of IRS-1 and calmodulin may be critical to intracellular insulin signaling.

CK2 and IkB - IkB encompasses a group of proteins which act to retain the NF-kB family of transcription factors in the cytoplasm. Cell activation ultimately leads to degradation of IkB which permits NF-kB to translocate to the nucleus and target gene activation. Stability of IkB is dependent on phosphorylation events on multiple residues located at the C and N termini. The N-terminus appears to be the target of inducible phosphorylation which marks IkB-α for degradation, while constitutive phosphorylation of its C-terminus "PEST" domain by CK2 is important for the intrinsic stability of IkB-α (177,178,179).

Altogether it appears that CK2 phosphorylation of IkB influences its stability, and therefore suggests that CK2 is important in regulating the transcriptional function of NF-kB, in the cytoplasm.

CK2 and PP2A - Purified PP2A has been shown to play an important role in down-regulating the MAPK pathway which is a pathway implicated in various cellular processes such as cell proliferation (180). PP2A was observed to interact *in vitro* with GST-CK2α, and to co-immunoprecipitate with endogenous CK2α from cell extracts of quiescent, but not platelet-derived growth factor PDGF stimulated, NIH-3T3 cells (87). The interaction between CK2α and PP2A resulted

in phosphorylation of a portion of the catalytic PP2A subunit (PP2Ac), and yielded a 30-50% stimulation of PP2A activity towards pre-phosphorylated MEK1 as substrate (87). Hériché et al., (1997), also observed that PP2A associated only with free CK2α, but not with CK2α associated with CK2β (87).

In cell culture assays, stable overexpression of CK2α in NIH-3T3 cells reduced cloning efficiency, while co-transfection of CK2α in RasV12 transformed cells, resulted in a 60% reduction in focus-formation assays (86). These results provide evidence for a negative role of CK2 in cell proliferation. This also implies that free CK2α could exist in cells and may function as a growth inhibitor through interaction and subsequent activation of PP2A, ultimately resulting in deactivation of the MAPK pathway in quiescent cells. These results are in sharp contrast to recent work reported by Orlandini et al., (1998) who observed that ectopic co-expression of CK2α or CK2α' with HA-ras, cooperated to increase focus formation in rat primary embryo fibroblasts (181). Moreover, transformed BALB/c 3T3 fibroblasts expressing HA-Ras and CK2α' displayed a faster growth rate (181). Further evidence that CK2α acts as a positive growth promoter was demonstrated in situations where overexpression of CK2α co-operated with c-Myc (182),

Tal-1 (183), or loss of p53 (184) in transgenic mice, resulted in accelerated development of T-cell lymphomas/leukemias. The findings of these latter reports are further discussed in the "CK2 the oncogene" section.

Overall, the bulk of the evidence supports a positive role for CK2α and CK2α' in cell growth and proliferation. However, as illustrated by the studies of Hériché et al., (1997), (87), there are discrepancies that need to be resolved.

OTHER PROTEINS -

The third and last group of CK2 substrates / protein partners is made up of interesting proteins which do not fit in groups 1 or 2. This group includes: Furin, $p21^{WAF1/CIP1}$, CD5, Fpr3, cdc37, and c-Mos.

CK2 and Furin - Furin is a membrane associated, calcium-dependent, serine endoprotease (185). Chapman and Munro, (1994), and Molloy et al., (1994), demonstrated that the 56 amino acid cytoplasmic tail of furin is important in its localization to the trans-Golgi Network (TGN) (186,187). Jones et al., (1995) demonstrated that the acidic cluster between furin residues 772-784 is essential for the targeting of furin to the TGN (100). Furthermore, CK2 phosphorylates furin at a pair of serine residues (Ser^{773} and

Ser^{775}) located within its cytoplasmic tail (100). Phosphorylation of these serine residues is not only critical for the localization of furin to the TGN, but is also important in modulating the retrieval of furin from the TGN (100). Dittié et al., (1997) further discovered that phosphorylation of furin by CK2 removes furin from immature secretory granules (IsGs), and enables furin to interact with the AP-1 adaptor complex (a component of the TGN) (188). Essentially, CK2 phosphorylation of furin molecules targets furin to the TGN, via the AP-1 adaptor complex.

CK2 and Mos - Mos is a germ cell-specific serine/threonine kinase whose activity is activated by progesterone stimulation, and is necessary for germinal vesicle breakdown (GVBD) in maturing oocytes (189,190). Chen et al., (1997) discovered that CK2β, interacts with Mos in a yeast-two hybrid system (126). These authors were able to demonstrate an association between CK2β and Mos (but not CK2α and Mos) in co-immunoprecipitates of *Xenopus* oocyte extracts, and that the interaction between CK2β and Mos inhibited Mos kinase activity *in vitro* (126). Furthermore, injection of Myc-tagged CK2β mRNA into immature *Xenopus* oocytes, resulted in a noticeable increase in CK2β protein levels, accompanied with a reduction in the sensitivity of

oocytes to progesterone (126). This phenomenon was shown to be a direct result of the CK2β protein, and not due to a change in CK2 activity (126). Interestingly, co-injection of CK2α and CK2β mRNA, could rescue the effect seen with CK2β. In fact, the 55 C-terminal amino acid residues of CK2β are necessary and sufficient to mediate this interaction (126). As noted earlier, the C-terminus of CK2β is important for CK2α and CK2β dimerization, suggesting a possible competition between CK2α and Mos for CK2β, in oocytes.

In summary,CK2β binding to Mos inhibits Mos kinase activity and prevents MKK/MAPK activation, which in turn prevents GVBD of oocytes. This supports a negative role for CK2β in cell maturation which can overcome by CK2α.

CK2 and p21$^{WAF1/CIP1}$ - p21$^{WAF1/CIP1}$ (p21) is a cyclin dependent kinase (cdk) inhibitory protein which acts as negative regulator of cell cycle progression at the G_1 stage of the cell cycle in a p53 dependent manner (191,192,193). Götz et al., (1996) demonstrated that p21 down regulates CK2 activity *in vitro* with respect to autophosphorylation, casein phosphorylation, and p53 phosphorylation, but is not appreciably phosphorylated by purified CK2 *in vitro* (129). Götz et al., (1996) further observed that p21 binds to CK2β,

but not CK2α, and that p21 competes with C-terminal p53 fragments for the binding to CK2β (129). This latter observation, implies a possible competition between p53 and p21 for CK2 in cells, and may reflect a key event involved in regulating the G_1 stage of the cell cycle.

The fact that p21 is a known negative regulator of cell cycle at G_1, suggests that a p21-directed inactivation of CK2 may be important for cellular G_1 arrest. Interestingly, the observation that CK2 possesses similarities with the cdks, (10), is consistent with the idea that some common regulation exists between CK2 and the Cdks. In fact, the interaction, and subsequent inactivation of CK2 by p21, may indicate another similarity, since p21 is known to interact and inhibit the activity of many cdks (192).

CK2 and CDC37 - Cdc37 was originally identified as a factor involved in the G_1 stage of the cell cycle (194), and was later observed to interact genetically with CK2α and CK2α' of *Saccharomyces cerevisiae* (12). Overexpression of CDC37 suppresses a deficiency in CK2 kinase activity, suggesting that Cdc37 may function as a kinase "activator" of CK2 (12), as well as other kinases such as cdc28 (195). Recently, it has been suggested that cdc37 is a kinase-targeting "subunit" of Hsp90 (196,197,198). Furthermore, Kimura et al., (1997), demonstrated that *in vitro*, Cdc37

stabilized mature, but unstable, CK2 at low salt concentrations (197). Although, the functional relationship between CK2 and Cdc37 remains undetermined, this is another example of the activity of CK2 regulated through its association with other proteins.

CK2 and CD5 - CD5 is a cell surface receptor present on all T cells and the B1 subset of B cells. Using the yeast-two hybrid system, CK2β was identified as an interaction partner for the cytoplasmic domain of CD5, and that the N-terminus of CK2β was critical for this interaction (124). Raman et al., (1998) further determined that neither CK2α nor CK2α' could interact with CD5, however CK2α is detected in CD5 immunoprecipitates (124). These same authors observed a CD5 specific stimulation of CK2 activity (9-10 fold) using anti-CD5 mAb (124).

The importance of these findings are twofold. First, this is yet another demonstration of modulation of CK2 activity through association with a protein. Secondly, this situates CK2 at the plasma membrane, and suggests an involvement for CK2 in membrane associated signaling.

CK2 and Fpr3 - A controversial report by Wilson et al., (1997) demonstrated that CK2 was able to phosphorylate a tyrosine residue (Tyr_{184}) of the nucleolar immunophilin Fpr3

in *Saccharomyces cerevisiae* (199). This tyrosyl phosphorylation involves a two step process which was dependent on an initial phosphorylation of a nearby Ser^{186} by CK2. The significance of the phosphorylation of Fpr3 by CK2 is unknown, but is of interest since this is the first evidence suggesting that CK2 may be a dual specificity kinase (i.e. able to phosphorylate serine/threonine and tyrosine residues). This finding is contradictory to reports in which CK2 was shown to be unable to phosphorylate Tyr residues (7,8).

The preceding section contained examples of functionally diverse CK2 substrates / protein partners, and serves to highlight the multifunctionality of CK2 in cells. I have reported that CK2 can regulate the activities of these various proteins not only by phosphorylation, but also by physical association and vice-versa. Moreover, the wide cellular distribution and large variety of these CK2 substrates / protein partners suggests that CK2 has specialized functions in specific cellular locations.

The following section will discuss the efforts of various laboratories to determine the cellular localization of CK2.

LOCALIZATION OF CK2 -

Active CK2 can be isolated from a variety of eukaryotic organisms, in the cytoplasm, nuclei and mitochondria (8). CK2 has also been found associated with the nuclear matrix, chromatin, and to a lesser extent, with core filaments and the cytoskeleton (133,200,201,202,203). The association of CK2 with the nuclear matrix and chromatin was observed to be an androgen dependent response, resulting in translocation of CK2 from the cytosol to the nucleus (200,201). The individual subunits of CK2 (CK2α, CK2α', and CK2β) have been detected in the cytosol of platelets using immunogold electron microscopy and Western blot analysis (204). Recently, a combination of biochemical fractionation, Western blot analysis and CK2 activity studies have demonstrated that the various CK2 subunits are associated with highly purified plasma membrane preparations (134).

Immunocytochemical studies using antibodies recognizing the CK2 holoenzyme have confirmed that CK2 resides in both the nucleus and the cytoplasm (205). CK2 has been reported to preferentially localize in the nucleus of proliferating cells, as compared to a general cellular, or cytoplasmic distribution, observed in quiescent cells (137,205). Investigation of the immunolocalization using specific CK2α and CK2β recognizing antibodies by Schmidt-Spaniol et al.,

(1993), demonstrated that CK2α and CK2β were predominantly present in both the nucleus of human primary fibroblasts, and human fibrosarcoma (HT1080)cells (206). However, in murine (3T3)cells, CK2α was found mostly in the cytoplasm, while CK2β was chiefly localized in the nucleus. Studies by Yu et al., (1991) aimed at examining the cellular localizations of all the CK2α, CK2α', and CK2β subunits in asynchronous, mitotic or G_1/S arrested HeLa cells, demonstrated that in asynchronous and hydroxyurea G_1/S arrested HeLa cells, there was a mainly cytoplasmic distribution of CK2α and CK2β, while CK2α' displayed a mainly nuclear localization (34). Furthermore, Yu et al., (1991), observed that the cellular distribution of CK2α' was influenced by the cell cycle stage in which the cells were in. It was reported that CK2α' is in the nucleus between the G_1 to S stages of the cell cycle, but is observed in the cytoplasm between S to G_2/M stages of the cell cycle(34). These observations lead to the speculation that the levels of CK2α' might be regulated in a cell cycle dependent manner, and/or there may be some shuttling of CK2α' between the nucleus and the cytoplasm in a cell cycle related manner (34). Moreover, in mitotic cells, Yu et al., (1991) observed a diffuse cytoplasmic CK2α signal, but a

strong CK2α signal associated with spindle fibers (34). Of note, in this study, is that the authors used antibodies able to specifically recognize CK2α, or CK2β, while the antibodies recognizing CK2α', also recognized CK2α. Therefore the authors determined the localization of CK2α' by "subtracting" the CK2α signal from the CK2α + CK2α' signal. To date, the observations by Yu et al., (1991) have not been confirmed using antibodies that specifically recognize CK2α'.

The most comprehensive study dealing with the cellular localization of the individual CK2 subunits was performed by Krek et al., (1992) (46). Using antibodies which specifically recognized each of the individual CK2 subunits, this group clearly demonstrated that the CK2 subunits are localized primarily to the nucleus, with some cytoplasmic staining, in primary chick embryo fibroblasts, or virally transformed chicken hepatoma cells (DU249) during interphase (46). Transiently expressed chicken CK2α or CK2β in human (HeLa) cells also displayed a predominantly nuclear localization (46). The results of these immunocytochemical studies were confirmed by fractionation of DU249 cells into cytoplasmic and nuclear fractions, followed by Western blot analysis of CK2α, CK2α', and CK2β (46). Interestingly, Krek et al., (1992) observed that although the three CK2 subunits

were distributed throughout the cell during mitosis, a fraction of both CK2α and CK2α' associated with the mitotic spindle, and CK2β co-localized with centrosomes (46). It is interesting to note that although these authors were able to detect and examine the endogenous CK2 subunits of highly proliferating chicken cells, and exogenous chicken CK2 in human (HeLa) cells, they failed to detect or examine endogenous CK2 in the HeLa cells, which Yu et al., (1991) were able to do (34,46). The reasons as to why one group, but not the other, was able to detect endogenous CK2 in HeLa cells is unknown, but may reflect differences in antibodies, or in methods of immunocytochemistry utilized.

In order to reconcile the observations by Yu et al., (1991) and Krek et al., (1992), Penner et al., (1997) overexpressed epitope-tagged human CK2α or CK2α' in Cos 7 cells and found that both CK2α and CK2α' are predominantly nuclear (58). This group also demonstrated that the kinase activities of either CK2α or CK2α' subunits are not required for the nuclear localization of CK2 (58).

An interesting report by Stigare et al., (1993) showed that the majority of nuclear CK2α, purified from the water midge *Chironomus tentans*, does not form heterooligomeric structures with CK2β (207). In fact, it appears that CK2α binds tightly to nuclear components, and further suggests

that CK2α may have some functions independent of CK2β, although this may be a feature specific to this organism.

In summary, it appears that CK2 is distributed throughout the cell, however discrepancies in CK2 localization exist. These discrepancies may simply reflect differences in cell lines, antibodies, or methods of sample preparation used, or perhaps, they may reflect a complex relationship between CK2 and its cellular location. However, it is evident that CK2 localization is not restricted to one cellular compartment. This view is supported by the identification of CK2 substrates and protein partners in the nucleus, cytoplasm, and plasma membrane.

CK2 IN CELLULAR PROLIFERATION AND CELL CYCLE PROGRESSION -

There is much existing evidence that suggests CK2 is an important player in cell proliferation. For instance, CK2 activity is elevated in rapidly growing cells, such as embryonic tissues (208), in proliferating cells compared to quiescent cells, and in tumour tissue versus non-tumour tissue (209,210). The importance of CK2 in cellular proliferation was highlighted when complete loss of CK2 activity was shown to be lethal to yeast and slime-mold (211,212,213).

Reports of modest levels of CK2 activation in response to insulin and epidermal growth (168,169,170,214), prompted

Carroll and Marshak, (1989) to examine the effects of cell growth stimulation on CK2 activity (215). In these studies, these authors serum-deprived human diploid fibroblasts (WI38), then examined the effects of serum-stimulated growth on CK2 activity (215). Carroll and Marshak, (1989), observed a rapid rise in CK2 activity (6-fold) 30 minutes after serum stimulation, followed by oscillations in CK2 activity over 32 hours post-serum stimulation (215). However, efforts by Schmidt-Spaniol et al., (1993), or Litchfield et al., (1994) to further these studies led to some different results (172,206). No major increases or oscillations of CK2 activity were observed in serum-stimulated or serum starved synchronized primary human fibroblasts.

A further role for CK2 in mitogenic signaling was investigated by several groups. Pepperkok et al., (1991) and Pyerin et al., (1992) were able to delay the entry of cells into S phase by pre-treating primary human fibroblasts (IMR-90) with antisense oligonucleotides directed against CK2α and CK2β (216,217). Microinjection of various specific CK2β antibodies inhibited serum-induced proliferation of IMR-90 cells (218). Furthermore, micro-injection of purified CK2 into quiescent cells was able to stimulate c-fos expression and cell proliferation (137). These same authors also observed a preferential relocation of CK2 within the nuclei

of rat embryo fibroblast(REF-52) cells upon serum stimulation, compared to a cytoplasmic localization of CK2 in serum starved cells.

Micro-injection of antibodies ,against CK2β, into the cytoplasm or the nucleus of Go-synchronized IMR-90 cells at the time of mitogenic stimulation or at various intervals thereafter, resulted in a delay of cell proliferation at different stages of the cell cycle (219). These results suggest that a certain level of tetrameric and/or active CK2 is necessary for the Go/G_1 and G_1/S transitions (219). Heller-Harrison and Czech (1991) showed that overexpression of CK2β in Cos 1 cells resulted in an increase in CK2 activity, indicating that a portion of CK2 (presumably CK2α or CK2α') is not fully active (39). This latter observation suggests there exists a pool of CK2 catalytic subunits which are not complexed in the CK2 holoenzyme or, alternatively, that a portion may be bound to a competitive inhibitor of CK2β (39).

The protein levels of the CK2α and CK2β subunits at different stages of the cell cycle have been examined by independent groups. Lorenz et al., (1993), examined the levels of CK2β in quiescent and exponential cells, while Schmidt-Spaniol et al., (1993) examined the levels of CK2α

and CK2β in both quiescent cells and exponentially growing cells (206,218). Schmidt-Spaniol et al., (1993) failed to notice any changes in levels of CK2α at different stages of the cell cycle, however both groups observed that quiescent cells contain less CK2β subunits than proliferating cells. These observations indicate that the transition of a cell from quiescence to proliferation is associated with an increase in protein levels of CK2β subunits (206,218). Recently, using differential screening to isolate transcripts induced by mitogenic stimuli within the G_1 phase, Orlandini et al., (1998), isolated and identified CK2α'(181). These authors also reported a slow and moderate induction in the message levels of all the CK2 subunits. CK2α' and CK2β mRNA levels showed a more pronounced increase (up to 1.5 fold), while CK2α rose modestly (1 to 1.25 fold), between 4-6 hours post-serum stimulation of serum starved NIH-3T3 cells (181). These authors also detected a concomitant modest increase in CK2 activity (1.5 fold) after 6 hours, however no major oscillations in CK2 activity were detected (181). Considering previous indications that the CK2α' protein is regulated in a cell-cycle dependent manner (34), a significant shortcoming, of all of the aforementioned studies, is that neither group examined the

protein levels of CK2α', leaving the need for a systematic examination of CK2α' expression during cell cycle progression. Therefore, in order to further and complete the studies on CK2 expression and activity during cell cycle progression, we examined CK2 activity and protein levels of CK2α, CK2α',CK2β in populations of transformed or primary cells collected at different stages of the cell cycle. The results are presented and discussed in Chapter 2 of this thesis.

Interesting observations were made from studies using *Saccharomyces cerevisiae* depleted of CK2 activity. Cells without CK2 activity arrested as a mixed population of budded and non-budded cells. A significant fraction of budded cells displayed an elongated morphology, which suggested that a lack of CK2 activity resulted in a cell cycle defect (211). Experiments with the temperature sensitive CK2α (cka1ts) or CK2α'(cka2ts) yeast cells grown at the restrictive temperature demonstrated that deprivation of cka2 activity resulted in two populations of cells arrested at either G_1 or G_2/M stages of the cell cycle (220). Recent experiments with temperature sensitive cka1ts cells grown at the restrictive temperature yielded a mixture of spherical unbudded and budded cells (221). These cells possessed non-polarized actin cytoskeleton as well as a significant fraction of multinucleate cell bodies. Similar

results were obtained by Snell and Nurse, (1994), with *Schizosaccharomyces pombe* (222). Upon characterization of morphological mutants, these authors identified a mutant displaying a spherical morphology. The phenotype of this particular mutant was attributed to the inability of the cells to re-establish polarized growth after cytokinesis. These authors further determined that this unusual phenotype was due to a temperature sensitive CK2α mutation in this *Schizosaccharomyces pombe* homologous mutant grown at the restrictive temperature (222). In a similar vein, it is interesting to note that another study was done in which overexpression of ckb1 in *Schizosaccharomyces pombe* cells, inhibited cell growth and cytokinesis, and resulted in multiseptation of cells (223).

Altogether, observations with mammalian cells demonstrate that CK2 is an important player in cell cycle progression, furthermore, studies of CK2 in yeast demonstrate that CK2 is important for cell viability, cell cycle progression and the maintenance of cell polarity.

CK2 THE "ONCOGENE" -

As previously discussed, CK2 phosphorylates proteins designated as oncogenic. Therefore, it should not be surprising to think of CK2 as possessing oncogenic properties.

An interesting observation was made by ole-MoiYoi, (1993) and ole-MoiYoi, (1995), in which the transcription, translation, and activity of CK2 are all increased in cattle afflicted with a leukemia-like disease known as "East Coast fever" (224,225). East Coast fever is caused by the parasite *Theileria parva* which infects bovine T-lymphocytes and eventually causes cell death by lymphocytolysis of these infected lymphocytes (224). As noted earlier, Orlandini et al., (1998), recently demonstrated that CK2α or CK2α' can cooperate with Ha-ras to transform rat embryo fibroblasts (181). Collectively these observations support the view that CK2 exhibits oncogenic activity. This notion was highlighted by using transgenic mice overexpressing human CK2α in the lymphoid organs of mice. Modest overexpression of CK2α resulted in a modest incidence of lymphoma which was inhibitable using CK2α antisense oligonucleotides (182). These results suggested that although CK2 expression appears to be important for proliferation of the tumor cells, however, other events are required for tumorogenesis. The tumorogenicity of CK2 was highlighted by the production of bi-hybrid transgenic mice co-expressing human c-myc and CK2α (182). These mice exhibited a marked increase in the rate of onset of fatal lymphoproliferative disease, as compared to the CK2α or c-myc overexpressing mice by

themselves, suggesting that cooperative tumorogenesis had occurred between c-myc and CK2α (182). Similar results were obtained with transgenic mice co-expressing Tal-1 and CK2α, in which these mice also exhibited an accelerated incidence of lymphoid malignancy (183). Recently, Landesman-Bollag et al., (1998) mated transgenic mice overexpressing CK2α with partially or completely p53 deficient transgenic mice (184). The resulting hybrids exhibited a marked acceleration in the formation of thymic lymphomas which was proportional to the level of functional p53. These experiments demonstrate that CK2α overexpression synergizes with loss of p53 to accelerate the development of lymphoma, but that another event is required for lymphomagenesis.

Overall, it is clear that although CK2α by itself may not be a potent transformation factor, when it is coupled with oncogenic factors or determinants, CK2α can dramatically accelerate the transformation process, thus supporting the view that CK2 is an "oncogene".

REGULATION OF CK2 ACTIVITY -

Compounds able to influence CK2 activity have been extensively studied *in vitro*. Polycationic compounds such as polyamine (e.g. spermine) and basic polypeptides (e.g.

polylysine or polyarginine) have been shown to stimulate CK2 activity (7,8). Polyamines are ubiquitous cellular components that are essential for cell proliferation and differentiation (226), while polylysine and polyarginine are artificial compounds. Intriguingly, unlike spermine, which increased the level of CK2β autophosphorylation, basic polypeptides prevented this CK2β phosphorylation (7). Furthermore, phosphorylation of calmodulin was increased with polylysine, but was not detected when CK2 was incubated with spermine (7). Experiments by several groups (38,227,228,229) have clearly demonstrated that spermine analogs bind to CK2β at an acidic stretch encompassing residues Asp^{51}-Tyr^{80}, and thus influence CK2 activity through the CK2β subunit.

Many polyanionic compounds have been identified as negative regulators of CK2 activity. The most potent inhibitor identified thus far is heparin. Heparin is an effective and specific inhibitor of CK2, with a Ki of $1.4x10^{-9}$ M, and has little effect on other protein kinases below 1 ug/mL (8). Inhibition by low concentrations of heparin, as well as the ability to utilize GTP, are properties which were commonly used for the identification of an unknown enzyme as CK2.

One limitation of these chemical activators and inhibitors is the physiological relevance of these

observations. For example, physiological activation by polylysine is unlikely, since polylysine is an artificial compound. However, physiological stimulation of CK2 by spermine is possible (Ka of 2.8×10^{-4} M), since this relatively high level of spermine can be found in several cell types such as red blood cells (230). Inhibition of CK2 by heparin may occur in tissues such as liver, where heparin is present in very small amounts (8).

Although regulation of CK2 activity by organic compounds/chemical inhibitors may be involved in the *in vivo* regulation of CK2, clearly these inhibitors and activators would only be able to regulate CK2 activity in specialized cells.

RESEARCH OVERVIEW

Clearly, CK2 is important for various aspects of cell proliferation and cell cycle progression. However, the precise mechanisms affecting the participation of CK2 participates in such important cellular events remains poorly understood.

In order to gain a better understanding of CK2 regulation and function in cells, my research specifically focused on the isozymic subunits (CK2α and CK2α') of CK2. As mentioned earlier, the CK2α and CK2α' subunits of vertebrates are very closely related (19). *In vitro* evidence suggests that CK2α and CK2α' are catalytically similar (33). However, the

observations that the amino acid sequences of CK2α and CK2α' differ completely at their unique CTDs (see Figs 1.1 and 1.2), and that differences between CK2α and CK2α' have been reported in cells (34,35), lead our laboratory to hypothesize that CK2α and CK2α' exhibit functional specialization in human cells, as do the two isozymic forms of CK2 in yeast (220,221). The research directed towards addressing this hypothesis is presented and discussed in chapters 2, 3, and 4 of this thesis.

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CHAPTER 2

EXPRESSION AND REGULATION OF PROTEIN KINASE CK2 DURING THE CELL CYCLE.

This work is currently in press in: *Mol. Cell. Biochem.*

Preface -

Observations by Yu et al., (1991), which described cell cycle related fluctuations in protein levels of CK2α and CK2α' in the nucleus and cytoplasm, suggested that CK2α and CK2α' may be actively shuttled during cell cycle progression, or alternatively, there was regulation of protein levels of CK2α and CK2α' in cells, in a cell cycle dependent manner (9). These observations, coupled with the importance of CK2 in cell cycle progression, led me to believe that differences between CK2α and CK2α' could manifest themselves through differential expression, or regulation, of CK2α and/or CK2α' in a cell cycle dependent manner. Until recently, reports in the literature have exclusively examined the CK2α and CK2β subunits, while ignoring CK2α', therefore the need to do a systematic study of the protein levels of all the isozymic subunits of CK2

during cell cycle, existed. In this regard, I examined and compared the relative expression levels of the CK2α and CK2α' subunits in primary and transformed cell lines at different stages of the cell cycle.

Contribution by the first author -

I performed all of the studies pertaining to the synchronized MANCA and GL30-92/R cells whose results are displayed in Fig. 2.1 and 2.2. I also wrote the "introduction", most of the "materials and methods" , and much of the "discussion" sections, under the editorial supervision of David Litchfield.

Expression and regulation of protein kinase CK2 during the cell cycle.

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ABSTRACT

There are indications from genetic, biochemical and cell biological studies that protein kinase CK2 (formerly casein kinase II) has a variety of functions at different stages in the cell cycle. To further characterize CK2 and its potential roles during cell cycle progression, one of the objectives of this study was to systematically examine the expression of all three subunits of CK2 at different stages in the cell cycle. To achieve this objective, we examined levels of CK2α, CK2α' and CK2β on immunoblots as well as CK2 activity in samples prepared from: i) elutriated populations of MANCA (Burkitt lymphoma) cells, ii) serum-stimulated GL30-92/R (primary human fibroblasts) cells and iii) drug-arrested chicken bursal lymphoma BK3A cells. On immunoblots, we observed a significant and co-ordinate increase in the expression of CK2α and CK2α' following serum stimulation of quiescent human fibroblasts. By comparison, no major fluctuations in CK2 activity were detected during any other stages during the cell cycle. Furthermore, we did not observe any dramatic differences between the relative levels of CK2α to CK2α' during different stages in the cell cycle. However, we observed a significant increase in the amount of CK2β relative to CK2α in cells arrested with nocodazole. We also examined the activity of CK2 in extracts or in immunoprecipitates prepared from drug-arrested cells.

Of particular interest is the observation that the activity of CK2 is not changed in nocodazole-arrested cells. Since CK2 is maximally phosphorylated in these cells, this result suggests that the phosphorylation of CK2 by $p34^{cdc2}$ does not affect the catalytic activity of CK2. However, the activity of CK2 was increased by incubation with $p34^{cdc2}$ *in vitro*. Since this activation was independent of ATP we speculate that $p34^{cdc2}$ may have an associated factor that stimulates CK2 activity. Collectively, the observations that relative levels of CK2β increase in mitotic cells, that CK2α and CK2β are phosphorylated in mitotic cells and that $p34^{cdc2}$ affects CK2 activity *in vitro* suggest that CK2 does have regulatory functions associated with cell division.

INTRODUCTION

Protein kinase CK2 (CK2) is a protein serine/threonine kinase that is ubiquitously distributed among eukaryotic cells. Although the exact role of CK2 in cells remains poorly understood, genetic and biochemical studies indicate that the enzyme is essential for viability and that it has important functions associated with cellular growth and proliferation (for review, see Refs. 1-4). CK2 is a heterotetrameric enzyme whose subunits exhibit a high degree of conservation between species (for review, see Refs. 1 and 5). In mammals, CK2 is comprised of two catalytic (α and/or α') subunits and two regulatory (β) subunits. Although they are closely related, CK2α and CK2α' are the products of distinct genes (6). In chickens and mammals, CK2α and CK2α' exhibit approximately 90% identity over their N-terminal 330 amino acids. By comparison, they differ markedly at their C-terminal domains (5). No functional differences between CK2α and CK2α' have been identified in cells. However, CK2α, but not CK2α', is phosphorylated in mitotic cells and in vitro by $p34^{cdc2}$ (7). We recently identified the mitotic phosphorylation sites of CK2α (8). Notably, these sites are all located within the unique C-terminal domain of CK2α. It has also been reported that the two forms of CK2 exhibit differential subcellular localization at different stages in

the cell cycle (9), although differences in the localization of CK2α to CK2α' have not been reported in subsequent studies (10,11).

The β subunit appears to stabilize and enhance CK2 activity, and also affects the substrate specificity of CK2 (12). Results from biosynthetic labelling studies in logarithmically growing cells indicate that CK2β is synthesized in excess of CK2α. However, unless it is complexed to CK2α or CK2α' most of the excess CK2β is rapidly degraded (13). There are also indications from a number of studies that CK2β is responsible for the formation of tetrameric CK2 complexes since two CK2β subunits have the ability to interact with each other (14-16). In cells, CK2β is phosphorylated at its autophosphorylation site, Ser^{2} and Ser^{3}, and at Ser^{209}, a site that is phosphorylated in vitro by $p34^{cdc2}$ (7, 17-19). The functional importance of these phosphorylation sites has not yet been elucidated.

There are a number of indications that CK2 plays an important role in cell cycle progression. Inactivation of CK2 in temperature-sensitive yeast strains resulted in cell cycle arrest at either the G_1/S or G_2/M transitions (20). In mammalian cells, antisense oligonucleotides or antibodies directed against CK2, inhibited progression through the G_0/G_1, and G_1/S transitions (21-24). Furthermore, Carroll and Marshak (25) reported oscillations of CK2 activity upon serum stimulation of human diploid lung fibroblasts.

Marshak and Russo (26) further investigated CK2 activity in human HeLa cell populations obtained at either G_1, S, or G_2/M stages of the cell cycle. CK2 activity was reported to decrease as cells progressed from the G_1 to G_2/M stages of the cell cycle. However, Schmidt-Spaniol et al. (27) performed similar experiments using primary human fibroblasts, but reported no major fluctuations in CK2 activity. These authors also looked at the α and β protein levels during progression from G_0 to G_2/M in primary human fibroblasts. In quiescent cells, they observed that the ratio between CK2α to CK2β was 10:1, whereas the ratio was 0.7:1 during the other stages of the cell cycle, suggesting that CK2β protein levels increase dramatically in proliferating cells.

As an extension of these previous studies and to resolve discrepancies regarding the cell cycle dependent expression of CK2, we have examined the expression of CK2α, CK2α' and CK2β as well as CK2 activity in: i) elutriated populations of MANCA (Burkitt lymphoma) cells, ii) drug-arrested chicken BK3A cells and iii) serum-stimulated GL30-92/R (primary human fibroblasts) cells. We have also examined the effect of $p34^{cdc2}$ on the activity of CK2. Overall, we have observed no major fluctuations in CK2 activity during different stages of the cell cycle. Furthermore, by Western blot analysis, we did not observe any dramatic alterations in the relative levels of CK2α to

CK2α' during different stages in the cell cycle, although we did observe a significant increase in the levels of both CK2α and CK2α' following serum stimulation of quiescent human fibroblasts. In extracts of human primary fibroblasts, CK2β was barely detectable and difficult to quantitate. By comparison, a strong CK2β signal was detected in MANCA cell extracts. Interestingly, we observed a significant increase in the amount of CK2β relative to CK2α in cells arrested with nocodazole. We also observed a striking increase in CK2 activity when the enzyme was incubated with $p34^{cdc2}$. Surprisingly, this activation appeared to be independent of phosphorylation.

MATERIALS AND METHODS

Materials-

Powdered RPMI 1640 and liquid D-MEM media were purchased from Gibco BRL. Fetal Bovine Serum used to culture the primary fibroblasts was obtained from Gibco BRL, while Fetal Bovine Serum (Fetalclone II) used to culture the MANCA cells was obtained from HyClone. Polyclonal antipeptide antibodies directed against the C terminus of CK2α (anti-$\alpha^{376-391}$), the C terminus of CK2α' (anti-$\alpha'^{333-350}$), the C terminus of CK2β (anti-$\beta^{198-215}$) or the C terminus of $p34^{cdc2}$ (anti-$p34^{cdc2\ 290-297}$) were previously described (7,17). Purified CK2 from bovine testis was obtained as previously described (7). Synthetic peptides, used as $p34^{cdc2}$ substrate [QLQLQAASNFKSPVKTIR] (17), and CK2 substrate [RRRDDDSDDD] (28), were synthesized on an Applied Biosystems Model 431A peptide synthesizer using Fmoc chemistry and were HPLC-purified on a C-18 reverse-phase column (Waters DeltaPak). ^{125}I-protein A was obtained from ICN. Rainbow coloured protein molecular weight markers, the ECL kit, and donkey anti-rabbit horseradish peroxidase were obtained from Amersham LIFE SCIENCE. Nocodazole, hydroxyurea and bovine serum albumin (BSA) were obtained from Sigma. Coomassie plus protein assay reagent was purchased from PIERCE. Phosphocellulose P81 paper was obtained from Whatman. Nitrocellulose membrane and pre-stained protein molecular

weight markers were obtained from Bio-Rad. Other chemicals and reagents were of reagent grade.

Centrifugal Elutriation-

Human B cells (MANCA, obtained from Dr. P. Whyte, McMaster University, Hamilton, Canada), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2mM glutamine in an atmosphere of 5% CO_2 and 95% air mixture at 37°C. Suspension cultures were maintained at a density of 2-5 x 10^5 cells/mL. For elutriation, MANCA cells (8 x 10^8) were collected by centrifugation and washed in ice-cold elutriation buffer (phosphate-buffered saline, pH 7.5 with 2mM EDTA). The cells were resuspended in 20 mLs of elutriation buffer and loaded into a Beckman JE-5.0 elutriation rotor (40 mL chamber) at a buffer flow rate of 95 mL/min, rotor speed of 2250 rpm, and rotor temperature of 4°C. Elutriation buffer (1L) was allowed to flow through the chamber before simultaneously decreasing the flow rate to 65 mL/min and rotor speed to 1650 rpm. Cell fractions were subsequently harvested by increasing the flow rate in a step-wise manner. Seven fractions (400 mL each) were collected on ice between buffer flow rates of 70 to 120 mL/min. Centrifugal elutriation enabled us to collect populations of cells between the G_1 -> G_2/M stages of the cell cycle as determined by flow cytometry.

Synchronization of fibroblasts by serum deprivation -

Primary human fibroblasts, GL30-92/R (obtained from Dr. S. Mai, Manitoba Institute of Cell Biology, Winnipeg, Canada), were grown, no later than passage 16, in D-MEM containing low glucose, L-glutamine, sodium pyruvate and pyridoxine hydrochloride, with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 and 95% air mixture at 37°C. Cells were grown to 25-30% confluence at which time, D-MEM medium containing FBS was replaced with D-MEM without FBS. Cells were incubated for 16 hours in the absence of FBS in order to make them quiescent. The D-MEM medium without FBS was then replaced with D-MEM containing FBS. Cells were collected at various times by trypsinization followed by extensive washing with cold phosphate-buffered saline, pH 7.5 with 2mM EDTA. This allowed us to collect population of cells between the G_0 -> G_2/M stages of the cell cycle as determined by flow cytometry.

Synchronization with hydroxyurea and nocodazole-

Chicken Bursal Lymphoma (BK3A) cells or human epidermal carcinoma (A431) cells were arrested at G_1/S using hydroxyurea and at mitosis using nocodazole as previously described (7,17).

Flow Cytometry -

For flow cytometric analysis, 3 x 10^6 cells from each fraction were washed and resuspended in 750 mL of ice-cold phosphate-buffered saline, pH 7.5 with 2mM EDTA. The cells were fixed by the gradual addition of 2 mL of 95% ethanol (-20°C) while vortexing. After 1-3 days at 4°C, the fixed cells were washed with elutriation buffer and resuspended in 1 mL of Propidium Iodide Solution (5 mg/mL propidium iodide, 1 mg/mL sodium citrate, 1 mg/mL Triton X-100, 50 U/mL RNase A). The cells were incubated on ice for 4 hours in darkness before measuring fluorescence intensities using an EPICS 753 fluorescence-activated cell sorter.

Preparation of cell extracts -

Cells were resuspended in extraction buffer (50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1mM Na_3VO_4, 1mM DTT, 10 mg/mL leupeptin, 10 mg/mL aprotonin, and 2 mg/mL pepstatin A) to a final concentration of 2-4 x 10^7 cells/mL. Cells were sonicated at half-maximal intensity and centrifuged at 100,000 x g for 20 minutes at 4°C. The supernatants were aliquoted and stored at -80°C.

Immunoblotting and quantitation of protein kinase CK2

subunits -

Protein concentrations of the cell extracts were determined using Coomassie plus protein assay reagent with bovine serum albumin as a standard. Aliquots of each cell extract (100 υg of protein) were subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose overnight at 30 Volts. For detection of CK2α, CK2α' and CK2β in MANCA cell extracts, anti-α and β antibodies were used at a concentration of 1/2000, while anti-α' antibodies were used at a concentration of 1/1000. For detection in GL30-92/R cell extracts, anti-α and anti-α' antibodies were added at a dilution of 1/500, while anti-β antibodies were added at a dilution of 1/125. Immunecomplexes were detected with ^{125}I-protein-A (1mCi/mL, final concentration). After extensive washing of the filters, and then drying, CK2 subunits were visualized and quantitated using a phosphoimager from Molecular Dynamics. Alternatively, immunecomplexes were detected by enhanced chemiluminescence using a 1/5000 dilution of Donkey Anti-rabbit horseradish peroxidase.

Kinase assays -

Protein kinase CK2 activity and $p34^{cdc2}$ activity were measured at 30°C in a 35 υL reaction mixture containing 10-30 mg of cell extracts in 50 mM Tris-Cl, pH 7.5, 10mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP (specific activity 400-1000 cpm/pmol) and 0.25 mM peptide (RRRDDDSDDD for CK2 activity; QLQLQAASNFKSPVKTIR for $p34^{cdc2}$ activity). Reactions were initiated by addition of cell extract, and terminated by spotting 30 υL of the reaction mixture on phosphocellulose P81 paper followed by washing in phosphoric acid (1%).

For measuring the effects of $p34^{cdc2}$ on CK2, $p34^{cdc2}$ was isolated from nocodazole arrested A431 cell extracts by immunoprecipitation in the presence or absence of competing antigenic peptide as previously described (7). Purified CK2, was incubated with immunopurified $p34^{cdc2}$ for 60 minutes at 30°C in the presence or absence of 0.1 mM ATP. CK2 was subsequently assayed using RRRDDDSDDD as substrate at 30°C in 25 mM Tris-Cl, pH 7.4, 10 mM MgCl2, 0.5 mM DTT, 0.2 mM ATP.

RESULTS

Expression and activity of CK2 in elutriated cells -

We were interested in examining the expression and activity of CK2 in cells at different stages of the cell cycle. To achieve this objective, we utilized centrifugal elutriation to fractionate populations of logarithmically growing cells into fractions with different cell cycle compositions. Elutriated populations of cells lack the level of synchrony achieved by drug treatment but deleterious effects of drugs are avoided. Flow cytometry (Figure 2.1A) was performed on the elutriated fractions of cells and demonstrates that separation of cells on the basis of cell cycle phase had been achieved. We subsequently examined the expression of the CK2α, CK2α', and CK2β subunits of CK2 from these elutriated MANCA cell extracts by Western blotting. The CK2 subunits were detected using specific polyclonal antibodies, and ^{125}I-protein-A (Figure 2.1, panels B and C). Each of the CK2 subunits were readily detected. Furthermore, the relative levels of CK2α,CK2α' and CK2β protein did not greatly fluctuate in the populations of cells collected at different stages of the cell cycle. Elutriated MANCA cell extracts were also assayed for both CK2 and $p34^{cdc2}$ activity (Figure 2.1, panel D). As observed with Western blots, there were no dramatic fluctuations in CK2 activity at different stages in the cell cycle. By

comparison, the activity of $p34^{cdc2}$ progressively increased in activity in successive fractions, exhibiting lowest activity in G_1 fractions and highest activity in the cell fractions with the G_2/M content. The increases in $p34^{cdc2}$ activity as a function of cell cycle progression reinforce the results of the FACS analysis, indicating that successful cell cycle fractionation had been achieved by centrifugal elutriation.

Expression and activity of CK2 in synchronized fibroblasts -

Since the MANCA cells that were subjected to centrifugal elutriation were logarithmically growing, we had not examined the expression or activity of CK2 during the events associated with cell cycle entry. Therefore, as an alternative to centrifugal elutriation, we performed serum stimulation of quiescent fibroblasts. Although synchrony following serum stimulation is not maintained for extended time periods, such an experiment is suitable for examining events that are associated with cell cycle entry and the early stages of cell cycle progression. Flow cytometric analysis shows that after serum deprivation treatment, greater than 90% of the human primary fibroblasts were in G_0/G_1 (Figure 2.2A). By comparison, at 27 hours after serum stimulation, the majority of these cells (greater than 60%) had progressed to G_2/M phase. Cell extracts obtained from these synchronized primary human fibroblasts (GL30-92/R)

were analyzed for expression of CK2α, CK2α', and CK2β subunits (Figure 2.2B,C), as well as CK2 activity (Figure 2.2D). The most notable observation is that there is a marked increase in the levels of both α and α' in cell extracts following serum stimulation. The protein levels, for both α and α' exhibited similar increases 2 hours after serum stimulation and then remained relatively constant as the cells continued to progress through S phase. In these fibroblasts, the levels of CK2β that were detected were very weak. Consequently, accurate quantitation was not possible. When we examined the activity of CK2 in these different cellular fractions, we did not observe any dramatic alterations in CK2 activity. On the basis of the results of the immunoblots, lower levels of CK2 activity in unstimulated cells may have been expected. We do not know why the results from immunoblots and from kinase activity measurements for the unstimulated cells are not well correlated to each other. However, these results suggest that cell extracts may contain factors that influence kinase activity measurements. We have not further explored the discrepancy between the kinase activity measurements and the Western blot analysis.

Expression and activity of CK2 in drug-arrested cells -

Although drug treatment may elicit undesirable effects including toxicity, a greater level of synchrony is achieved than with elutriation or by serum-stimulation of quiescent cells. Furthermore, drug treatment may allow an examination of cells that are arrested at stages within the cell cycle that are difficult to attain in cycling populations because of their transient nature. Therefore, to complement our analysis of CK2 activity and expression in cells that are progressing through the cell cycle, we also examined the activity and expression of CK2 in cells arrested with hydroxyurea (G_1/S arrest) or with Nocodazole (M phase arrest). As previously observed in cells arrested with nocodazole, CK2α exhibits a retarded electrophoretic mobility. We have previously demonstrated that this altered mobility is the result of phosphorylation by $p34^{cdc2}$ (7,8). Quantitation of the amount of CK2α that is present in the different samples indicates that there are no dramatic alterations in the total levels of CK2α in hydroxyurea or nocodazole-arrested cells as compared to untreated cells. By comparison, there is a significant (approximately 2-fold) increase in the levels of CK2β in the extracts from the nocodazole-arrested cells.

We also examined the activity of CK2 in immunoprecipitates (Figure 2.3B) or in extracts (Figure 2.3C) prepared from the different cell populations. As

expected in cell extracts, we observe a dramatic activation in $p34^{cdc2}$ activity in nocodazole-arrested cells (Figure 2.3C). By comparison dramatic alterations in CK2 activity are not observed in cell extracts or in immunoprecipitates. Since CK2α and CK2β are dramatically phosphorylated in mitotic cells, these results suggest that the activity of CK2 is not affected by this phosphorylation.

Activation of CK2 by $p34^{cdc2}$ -

As previously shown (7,8), and as noted above (Figure 3C), CK2 is phosphorylated in mitotic cells. We did not observe any changes in the activity of CK2 that had been phosphorylated in mitotic cells. However, there had been reports from Muller-Lorillon et al. (29) and from Meggio et al. (30) that CK2 is activated *in vitro* by $p34^{cdc2}$. The latter study suggested that activation of CK2 was independent of its phosphorylation by $p34^{cdc2}$. We were therefore interested in examining the activity of CK2 following incubation with $p34^{cdc2}$. For this purpose, purified tetrameric CK2 was incubated with immunopurified $p34^{cdc2}$, or as a control, with immunoprecipitates of $p34^{cdc2}$ that were performed in the presence of an excess of antigenic peptide. Furthermore, we incubated CK2 with $p34^{cdc2}$ or the control immunoprecipitates in the presence or absence of ATP. As shown in Figure 2.4, pre-incubation of CK2 with $p34^{cdc2}$ resulted in a significant increase in CK2 activity as

compared to the CK2 that had been pre-incubated with control immunoprecipitates. This apparent activation of CK2 by $p34^{cdc2}$ occurred in the absence of ATP, a result that is consistent with the suggestion of Meggio et al., (30) that the activation of CK2 by $p34^{cdc2}$ is independent of phosphorylation. There was no effect when CK2 was pretreated with alkaline phosphatase prior to incubation with $p34^{cdc2}$ (data not shown).

DISCUSSION

In this paper, we present data on the expression and activity of CK2 during different stages of the cell cycle. For these studies, cells at different stages in the cell cycle were obtained by centrifugal elutriation, by serum stimulation of serum-deprived cells and by drug arrest. By centrifugal elutriation we were able to examine populations of cells at different stages in the cell cycle that were isolated from a population of cycling cells. Serum-stimulation of quiescent cells complemented this analysis by permitting an examination of CK2 activity and expression during the early events of cell cycle entry and progression. Finally, drug treatment permitted an examination of cells that were arrested at stages in the cell cycle (ie. G_1/S or in mitosis) that are normally difficult to attain because of their transient nature.

In agreement with a number of studies (2, 21-24, 31) we observed an increase in the levels of CK2 expression following stimulation of quiescent cells to re-enter the cell cycle. We did not observe any differences in the relative expressions of CK2α and CK2α', as the expression of both isoforms increased following stimulation. Although we observed an increase in immunoreactive CK2 following serum stimulation, we did not observe a similar increase in CK2 activity in extracts. We do not have any firm explanations

for this observation. However, this observation may, at least in part, provide an explanation for the problems associated with characterization of CK2 activity following treatment of cells with a variety of stimuli (discussed in Litchfield et al., 40). Furthermore, this observation does point out the perils of using a single technique (ie. immunoblotting or activity measurements) when assessing the role of CK2 in cellular responses. In this study, we have also observed a significant difference in the basal specific activity of CK2 between the human primary fibroblasts (GL30-92/R) and the human lymphoma cells (MANCA). Levels of CK2 subunits were also more difficult to examine in the primary fibroblasts than in the MANCA cell extracts. In fact, less dilute antibody concentrations were necessary for detection of proteins in fibroblast extracts.

In cycling cells (as compared to quiescent cells that have been stimulated to re-enter the cell cycle) we do not observe major fluctuations in CK2 activity or expression at different stages of the cell cycle. Furthermore, we did not detect any differences in the activity of CK2 in drug-arrested cells. Overall, our data agree with the observations made by Schmidt-Spaniol et al., (27), in which they also found no major oscillations in CK2 activity during the progression from G_0 through to the G_2/M transition in human primary fibroblasts (HT1080). By comparison, dramatic oscillations in CK2 activity were reported by Carroll and

Marshak (25) using human lung fibroblasts (WI-38). The differences in results may simply reflect differences in the cell types used, differences in the times at which cells were collected or assay conditions. However, these discrepancies once again underscore the lack of certainty concerning the regulation of CK2 and its activity in cells (40).

Although there were no apparent alterations in the expression of CK2β in elutriated cells or in serum-stimulated fibroblasts, we have observed a significant increase in the expression of CK2β (relative to CK2α) in nocodazole-arrested cells. In view of our observation that CK2β which does not form a complex with CK2α is rapidly degraded (13), this result may suggest that the mechanisms responsible for the degradation of CK2β may be altered in mitotic cells. As noted by Allende and Allende (1), it may be of interest that CK2β contains a sequence resembling the destruction box of mitotic cyclins that is required for ubiquitin-dependent degradation in a cell cycle specific manner. We have shown that CK2β is degraded by a non-lysosomal process, that it is ubiquitinated in cells (data not shown), and we are currently exploring the role of the putative destruction box in controlling the stability of CK2β in cells.

Based on the increase in levels of CK2β relative to CK2α in mitotic cells, it is important to consider the

possibility that the individual subunits of CK2 have functions that are independent of the CK2 holoenzyme. In this regard, it is of interest that CK2β has been shown to interact in cells with the catalytic subunits of other protein kinases including c-Mos (32) and A-raf (33-34). It is particularly intriguing that CK2β appears to play a role in inhibiting the activity of c-Mos in oocytes and in preventing the ability of Mos to promote oocyte maturation. Down-regulation of CK2β alleviates this inhibition and promotes oocyte maturation (32). Based on our observation that CK2β increases in mitotic somatic cells, it is tempting to speculate that CK2β may also have a role in the regulation of mitotic events. The observations of Roussou and Draetta, (35) who demonstrated that overexpression of the CK2β subunit in *Schizosaccharomyces pombe* inhibited cell growth and cytokinesis may in fact be consistent with this suggestion. Furthermore, the localization of CK2β, but not CK2α, to centrosomes (10), may also be consistent with a role for CK2β has a role in the regulation of mitotic events. Our previous observation that CK2α and CK2β are phosphorylated by $p34^{cdc2}$ *in vitro* and in mitotic cells provides additional evidence that CK2 may be involved in various aspects of cell division. $P34^{cdc2}$ is essential for cell division; activation of $p34^{cdc2}$ initiates a massive burst of protein phosphorylation that is associated with a variety of cellular alterations (ie. chromosome

condensation, nuclear envelope breakdown, nucleolar disassembly, cytoskeletal reorganization etc.) (for review, see Ref: 36). Interestingly, CK2 is known to phosphorylate a wide variety of proteins including chromosome-associated proteins, nucleolar proteins and cytoskeletal proteins. Furthermore, CK2 has been localized to the mitotic spindle (9,10). Collectively, these observations suggest that $p34^{cdc2}$ may regulate CK2 in cells and that CK2 is an active participant in the burst of phosphorylation that accompanies the onset of mitotis (Figure 2.5). The observation by Cardenas et al. (37) that the mitotic phosphorylation sites of topoisomerase II are not phosphorylated at the non-permissive temperature in yeast with a temperature-sensitive form of CK2 supports this hypothesis.

To explore how CK2 could be regulated by $p34^{cdc2}$, we examined the activity of CK2 in extracts or immunoprecipitates prepared from mitotic cells. Our results indicate that the phosphorylation of CK2 by $p34^{cdc2}$ does not directly influence the activity of CK2. However, *in vitro* experiments aimed at addressing this issue showed that incubation of purified CK2 with $p34^{cdc2}$ increased CK2 activity. Surprisingly, as previously noted by Meggio et al. (30), this effect appeared to be independent of phosphorylation. One possibility is that the preparations of $p34^{cdc2}$ may contain an associated factor that is capable of activating, or at least stabilizing CK2. Although we do not

have any data concerning this issue, one potential candidate may be a molecular chaperone such as Cdc37. Cdc37 has recently been shown to be capable of activating a number of protein kinases including CK2 (38). Interestingly, Cdc37 was also identified as a suppressor for loss of CK2 function in yeast suggesting that there may in fact be a genetic linkage between CK2 and Cdc37 (39). Furthermore, a mammalian homolog of Cdc37 has been identified as a factor that associates with, and activates, CDK4. Whether Cdc37, or a related factor, is responsible for the activation of CK2 by $p34^{cdc2}$ remains to be determined. Clearly, elucidation of the mechanism by which CK2 is activated by $p34^{cdc2}$ *in vitro* may provide insights for defining the regulation of CK2 in cells.

Overall, we have not observed any dramatic fluctuations in the activity of CK2 during the cell cycle. However, we did observe a significant increase in the expression of CK2 when cells are stimulated to re-enter the cell cycle. Furthermore, we observed a marked increase in the levels of CK2β in cells arrested in mitosis. These findings, and our previous observation that CK2 is a target for $p34^{cdc2}$ *in vitro* and in mitotic cells, may reflect important functions of CK2 for entry into and exit from the cell cycle.

ACKNOWLEDGEMENTS

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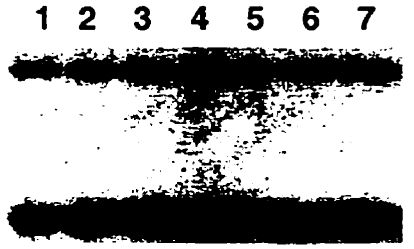
Fig. 2.1. **CK2 expression and activity in synchronized MANCA cells**. Synchronous populations of Burkitt Lymphoma cells were collected at different stages of the cell cycle using centrifugal elutriation. A) Cell Synchrony was assessed by flow cytometric analysis as described in "Materials and Methods" section. Cell extracts (100 υg protein), prepared as described in "Materials and Methods", were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and blotted onto Nitrocellulose membrane followed by immunodetection of CK2α (upper band) and CK2β (lower band)(B), or CK2α' (upper band) and CK2β (lower band)(C). The blots were developed with ^{125}I protein-A and visualized by autoradiography. D) CK2 specific activity (open boxes) was measured using the synthetic peptide RRRDDDSDDD, while $p34^{cdc2}$ activity (closed boxes) was measured using the synthetic peptide QLQLQAASNFKSPVKTIR as described in "Materials and Methods".

A

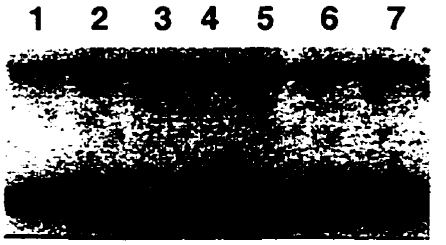
Fraction 1 Fraction 4 Fraction 7

B



CK2α and CK2β

C



CK2α' and CK2β

D

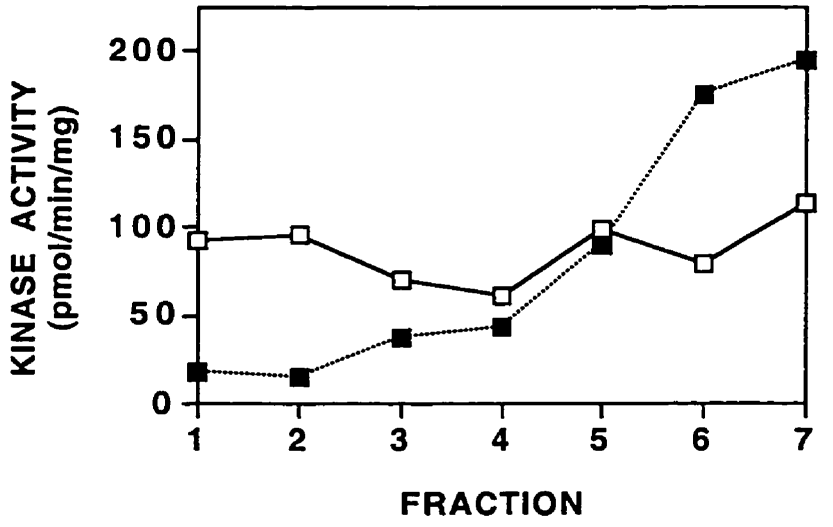
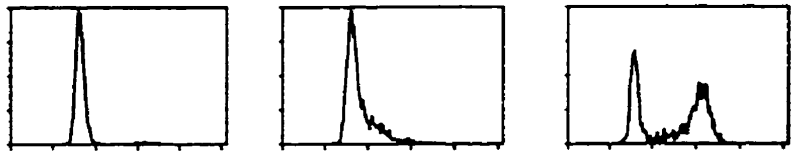
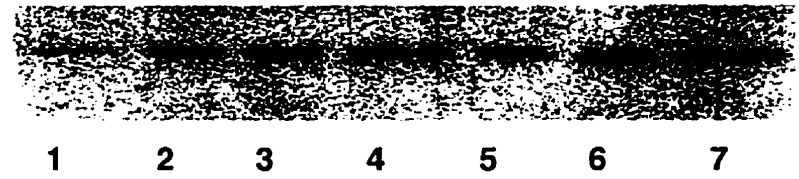


Fig. 2.2. **CK2 expression and activity in synchronized primary fibroblasts**. Human primary fibroblasts (GL30-92/R) were synchronized at Go by serum deprivation and induced to re-enter the cell cycle by addition of serum. Cells were harvested at various time points. A)Cell Synchrony was assessed by flow cytometric analysis as described in "Materials and Methods" section. Cell extracts (100 υg protein), prepared as described in "Materials and Methods", were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and blotted onto Nitrocellulose membrane followed by immunodetection of CK2α (B), or CK2α' (C). The blots were developed using ECL and exposed to film. D) CK2 specific activity was measured using the synthetic peptide RRRDDDSDDD.

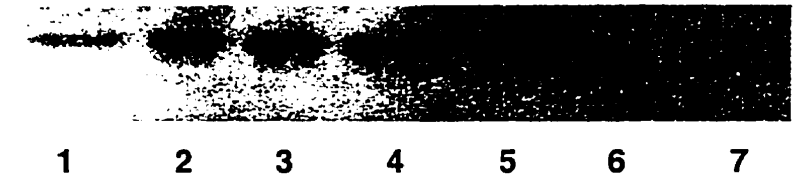
A


Fraction 1
Fraction 6
Fraction 7

B


1
2
3
4
5
6
7

C


1
2
3
4
5
6
7

D

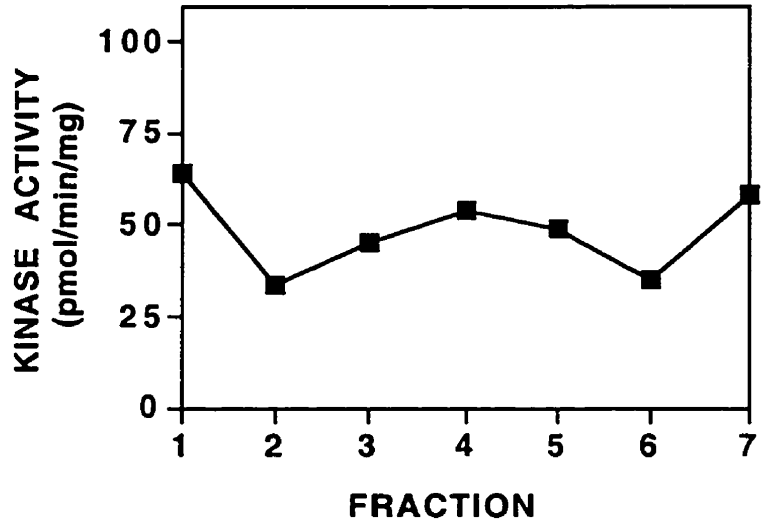
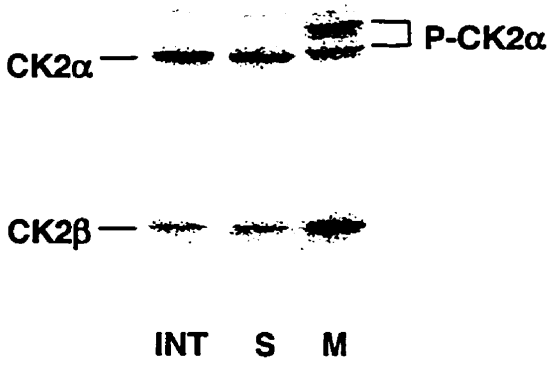

KINASE ACTIVITY
(pmol/min/mg)
100
75
50
25
0
1
2
3
4
5
6
7
FRACTION

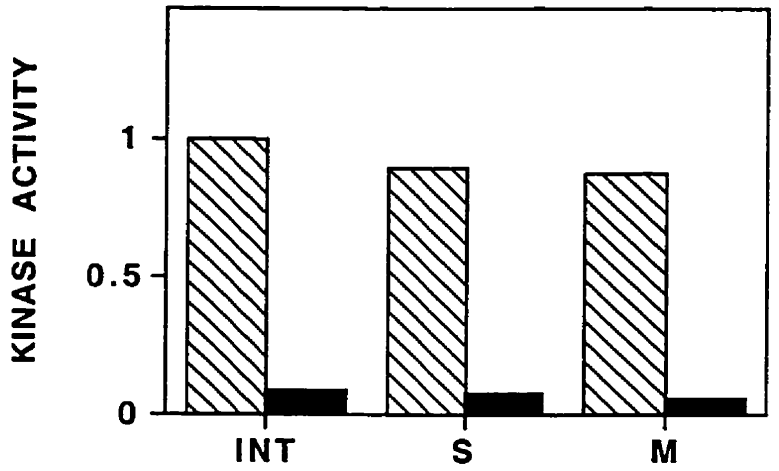
Fig. 2.3 **CK2 expression and activity in drug arrested chicken BK3A cells.**

Chicken bursal lymphoma cells were grown logarithmically (INT), or arrested with hydroxyurea (S) or with nocodazole (M) as previously described (7). Extracts were prepared from these cells and analysed for the expression of CK2α and CK2β on an immunoblot (panel A) using ^{125}I-protein A to detect immunecomplexes (50 υg of protein per lane). The positions of CK2α and CK2β as well as the phosphorylated forms of CK2α (P-CK2α) are indicated. B) CK2 activity was measured in immunoprecipitates prepared from each of the cell extracts with anti-CK2β$^{198-215}$ antibodies in the presence (solid bars) or absence (hatched bars) of competing antigenic peptide. C) CK2 activity (hatched bars) or p34^{cdc2} (solid bars) were measured in extracts using the synthetic peptides RRRDDDSDDD (CK2) or QLQLQAASNFKSPVKTIR (p34^{cdc2}) as substrate.

A


CK2α
P-CK2α
CK2β
INT
S
M

B


KINASE ACTIVITY
1
0.5
0
INT
S
M

C

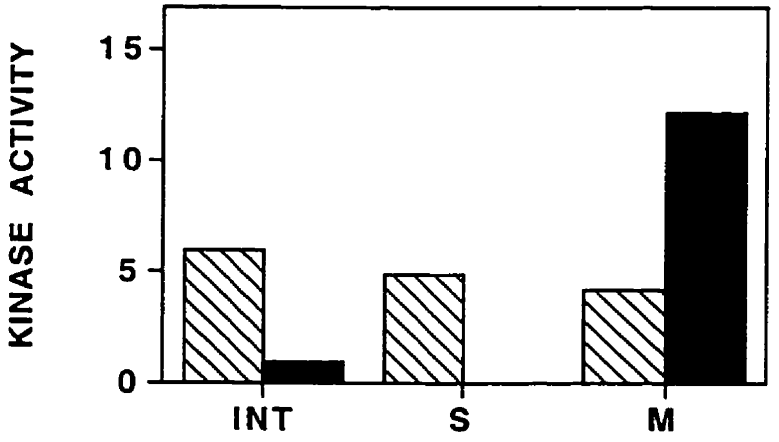

KINASE ACTIVITY
15
10
5
0
INT
S
M

Fig. 2.4 **The activity of CK2 is increased by $p34^{cdc2}$** .
$P34^{cdc2}$ was isolated from nocodazole-arrested cells by immunoprecipitation using anti-$p34^{cdc2}$ antibodies in the presence (marked with - for cdc2) or absence (marked with + for cdc2) of antigenic peptide. Purified CK2 was incubated with the immunecomplexes as described in " Materials and Methods " in the presence (+) or absence (-) of ATP prior to measurement of CK2 activity.

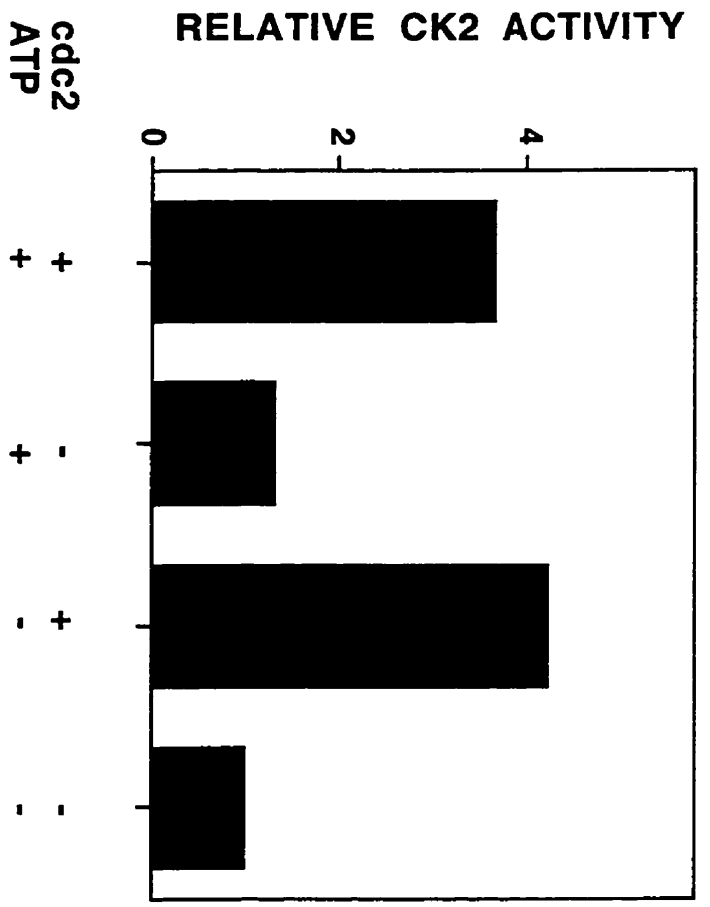

RELATIVE CK2 ACTIVITY
0
2
4
cdc2
ATP
+ +
+ -
- +
- -

Fig. 2.5 **Activation of $p34^{cdc2}$ triggers a massive burst of phosphorylation**. Activation of $p34^{cdc2}$ at the initiation of mitosis initiates a series of dramatic cellular alterations that are associated with a burst of protein phosphorylation. The observation that CK2 is phosphorylated by $p34^{cdc2}$ in mitotic cells suggests that CK2 is a regulatory participant in events associated with cell division.

p34cdc2
CK2
other PKs
nuclear disassembly
cytoskeletal rearrangement
mitotic spindle formation
transcriptional termination
translational termination

CHAPTER 3

PHOSPHORYLATION OF CASEIN KINASE II BY $p34^{cdc2}$: IDENTIFICATION OF PHOSPHORYLATION SITES USING PHOSPHORYLATION SITE MUTANTS IN VITRO.

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Preface -

The important observation by (Litchfield et al., 1992) that CK2α, but not CK2α' is phosphorylated in mitotically arrested cells (21), implies that CK2α and CK2α' are regulated differently in cells, which in turn may reflect the existence of specialized functions between the two isozymes. Furthermore, this mitotic phosphorylation of CK2 may play a crucial role in regulation of CK2 function and may be an integral event in mitosis.

An important first step in being able to characterize the mitotic phosphorylation of CK2α, is to identify the mitotic sites of phosphorylation. Identification of these sites is critical to the production and the subsequent characterization of phosphorylation-site mutants in cells, which should enable us to define the role of CK2 and its phosphorylation during cell division.

In this chapter, I describe how our laboratory endeavoured to successfully identify the mitotic sites of phosphorylation of CK2α.

Contribution by the first author -

I purified all of the GST-fusion proteins, and performed all of the phosphorylation assays with these fusion proteins, as well as with the synthetic peptides. I produced all of the phosphopeptide maps, did the phospho-amino acid analysis, and prepared the associated figures. I wrote the "materials and methods" section, and contributed to the "introduction" , "results" , and "discussion" sections, with editorial input from David Litchfield.

Phosphorylation of Casein Kinase II by $p34^{cdc2}$: Identification of Phosphorylation Sites using phosphorylation site mutants *in vitro*

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Running Title: Mitotic phosphorylation sites on casein kinase II

SUMMARY

The α and β subunits of casein kinase II are dramatically phosphorylated in cells that are arrested in mitosis (Litchfield, D.W., Lüscher, B., Lozeman, F.J., Eisenman, R.N. and Krebs, E.G. (1992) *J. Biol. Chem.* **267**: 13943-13951). Comparative phosphopeptide mapping experiments indicated that the mitotic phosphorylation sites on the α subunit of casein kinase II can be phosphorylated *in vitro* by $p34^{cdc2}$. In the present study, we have demonstrated that a glutathione S-transferase fusion protein encoding the C-terminal 126 amino acids of the α subunit is phosphorylated by $p34^{cdc2}$ at the same sites as intact casein kinase II indicating that the mitotic phosphorylation sites are localized within the C-terminal domain of α. Four residues within this domain, Thr-344, Thr-360, Ser-362 and Ser-370, conform to the minimal consensus sequence for $p34^{cdc2}$ phosphorylation. Synthetic peptides corresponding to regions of α that contain each of these residues are phosphorylated by $p34^{cdc2}$ at these sites. Furthermore, alterations in the phosphorylation of the glutathione S-transferase proteins encoding the C-terminal domain of α are observed when any of the four residues are mutated to alanine. When all four residues are mutated to alanine, the fusion proteins are no

longer phosphorylated by $p34^{cdc2}$ at any of the sites that are phosphorylated in mitotic cells. These results indicate that Thr-344, Thr-360, Ser-362 and Ser-370 are the sites on the α subunit of casein kinase II that are phosphorylated in mitotic cells.

INTRODUCTION

Biochemical and genetic studies have demonstrated that the $p34^{cdc2}$ protein kinase is an indispensible regulator of events leading to the division of eukaryotic cells (for reviews see 1-4). To ensure that the division of cells is very precisely regulated, the activity of this protein serine/threonine kinase is exquisitely controlled through its interactions with regulatory cyclins and through phosphorylation of $p34^{cdc2}$ itself. $P34^{cdc2}$ is defined as a cyclin dependent kinase (CDK) since it is inactive unless it is associated with a regulatory cyclin subunit. Furthermore, $p34^{cdc2}$ is inhibited by phosphorylation of Tyr-15 and/or Thr-14 (5,6), but requires phosphorylation of Thr-161 in order to be activated (7,8). CAK (the $p34^{cdc2}$ activating kinase) is responsible for phosphorylation of Thr-161 (9-11), while the phosphorylation state of Thr-14 and/or Tyr-15 is at least in part controlled by the relative activities of the *Wee1* protein kinase (12,13) and *cdc25* protein phosphatase (14,15).

Concomitant with the activation of $p34^{cdc2}$ at the G_2/M transition of eukaryotic cells is a massive burst of protein phosphorylation. Many of the events that are associated with entry into mitosis including nuclear envelope breakdown, transcriptional termination, nucleolar disassembly, cytoskeletal reorganization and chromosome condensation

appear to be associated with protein phosphorylation. While it is evident that $p34^{cdc2}$ directly phosphorylates a number of proteins at the G_2/M transition, there are also indications that $p34^{cdc2}$ could indirectly regulate phosphorylation events through its phosphorylation of other protein kinases (16-23).

One protein serine/threonine kinase that could be regulated by $p34^{cdc2}$ is casein kinase II (CKII) which has been shown to be dramatically phosphorylated in mitotic cells (19-21). CKII is a messenger-independent enzyme composed of two catalytic (α and/or α' subunits) and two additional subunits (β subunits) (for reviews see 24-26). Our previous studies demonstrated that $p34^{cdc2}$ phosphorylates the β subunit of CKII at Ser-209, a site that is maximally phosphorylated in mitotic cells (20). Interestingly, the α subunit (but not the α' subunit) of CKII is also dramatically phosphorylated in mitotic avian and mammalian cells (21). This result suggests that there may be differences in the functional or regulatory properties of the isozymic forms of the catalytic subunit of CKII. Our analyses demonstrated that the mitotic phosphorylation sites on a can be phosphorylated *in vitro* by $p34^{cdc2}$. To facilitate efforts to examine the functions of CKII during mitosis and how phosphorylation may affect these functions, the objective of the present study was directed towards

identification of the sites on the α subunit of CKII that are phosphorylated by $p34^{cdc2}$ so that non-phosphorylatable forms of CKII could be prepared by mutagenesis. Utilizing synthetic peptides and glutathione S-transferase (GST) fusion proteins containing the carboxy terminal domain of the human CKII α subunit, $p34^{cdc2}$ phosphorylation sites were identified as Thr-344, Thr-360, Ser-362 and Ser-370. Furthermore, following mutation of each of these residues to alanine residues, fusion proteins containing the carboxy terminal domain of CKII α were no longer phosphorylated by $p34^{cdc2}$ at any of the sites that are phosphorylated in mitotic cells.

EXPERIMENTAL PROCEDURES

Materials -

Synthetic peptides (Peptide 1: Pro-Gly-Gly-Ser-Thr-Pro-Val-Ser-Ser-Ala; Peptide 2: Ile-Ser-Ser-Val-Pro-Thr-Pro-Ser-Pro-Leu-Gly-Pro-Leu-Ala-Gly; Peptide 3: Ile-Ser-Ser-Val-Pro-Thr-Pro-Ala-Pro-Leu-Gly-Pro-Leu-Ala-Gly; Peptide 4: Leu-Gly-Pro-Leu-Ala-Gly-Ser-Pro-Val-Ile-Ala-Ala) were synthesized with an Applied Biosystems Model 431A Peptide Synthesizer using Fmoc chemistry and were purified by reverse phase HPLC using a C18 column as previously described (20). Polyclonal antipeptide antibodies directed against the C-terminus of CKII α (anti-$\alpha^{376-391}$) and the C-terminus of CKII β (anti-$\beta^{198-215}$) were previously described (20,21). $P34^{cdc2}$ was purified from nocodazole-arrested MANCA cells as previously described (27) and had a specific activity of approximately 300 nmol/min/mg when assayed at 30°C using histone H1 as substrate. [γ-^{32}P]ATP was obtained from Du Pont-New England Nuclear. Thermolysin was obtained from Boehringer Mannheim. The Muta-Gene M13 In Vitro mutagenesis Kit, version 2 and the Bst DNA sequencing kit were obtained from Bio-Rad. Restriction enzymes and T4 DNA ligase were from Pharmacia. Protein A-Sepharose, glutathione agarose, glutathione and nocodazole were obtained from Sigma. Thin layer cellulose plates (0.1 mm) for phosphopeptide mapping and phosphoamino acid analysis were

from Merck. Other chemicals and reagents were of reagent grade.

Methods

Expression of GST Fusion Proteins -

The cDNAs encoding the human CKII α and human CKII α' subunits (28) were obtained as clones which had been cloned into the *Bam*H1 site of pBluescript SK+. To express GST fusion proteins containing the C-terminal domain of CKII α (GST-αC126), a 629-base pair *Sau3*A1 fragment of the human CKII α cDNA, encoding the C-terminal 126 amino acids of CKII α, was cloned into the *Bam*H1 site of the pGEX-1 vector (29) to make the pGEX-1/CKIIα construct. To express a fusion protein containing the C-terminal domain of CKII α' (GST-α'C51), a 185 bp *Bgl*II/*Bam*H1 fragment of the human CKII α' cDNA, encoding the C-terminal 51 amino acids of CKII α' was similarly cloned into the *Bam*H1 site of pGEX-1 to make the pGEX-1/CKII α' construct. Site-directed mutants of GSTαC126 were prepared by the method of Kunkel (30) using a Muta-Gene M13 In Vitro Mutagenesis Kit (Biorad) according to the Manufacturer's recommendations. Briefly, a 426 bp fragment encoding the C-terminal region of CKII α was obtained by digestion of the pBluescript SK+/CKII α plasmid with *Sph*1

(at nucleotide 1174 within the CKII α cDNA) and with *Eco*R1 (within the multiple cloning site of pBluescript SK+) and cloned into M13 mp18. Single stranded phage DNA was prepared and used as template for oligonucleotide-directed mutagenesis using the following oligonucleotides: 5'AGG GGG CAG TGC GCC CGT CA 3' (for Thr-344/Ala), 5'CAG TGC CAG CCC CTT CA 3' (for Thr-360/Ala), 5' CAA CCC CTG CAC CCC TT 3' (for Ser-362/Ala), 5' TGG CAG GCG CAC CAG TG 3' (for Ser-370/Ala) and 5' CAG TGC CAG CCC CTG CA 3' (for Thr-360/Ala and Ser-362/Ala). Mutants were identified by DNA sequencing of single stranded templates using the dideoxy method of Sanger et al. (31) with a Bst Sequencing Kit (Biorad). DNA sequencing also confirmed that no additional mutations were introduced. Subsequent rounds of mutagenesis were performed to obtain constructs with mutations at multiple sites. Following preparation of the various mutants, the *Sph*1/*Eco*R1 fragments were cloned into the pGEX-1/CKII α construct encoding GST-αC126 in order to express fusion proteins harboring mutations at the putative phosphorylation sites.

Fusion proteins were expressed in *Escherichia coli* JM109 and purified using glutathione-agarose as described previously (29). Briefly, fresh overnight cultures of transformed JM109 cells, in Luria-Bertani (LB) medium (50 mL) containing ampicillin (50μg/mL), were diluted 1:10 in fresh medium and incubated for 1 hour at 37°C with shaking.

Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM and cells incubated for an additional three hours. Cells were pelleted by centrifugation at 5 000 X g and resuspended in 1/10th volume of NET (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1mM EDTA). Cells were then lysed on ice using mild sonication (3 X 10 seconds) and centrifuged at 10 000 X g. Supernatants were tumbled for 1 hour at 4°C with glutathione-agarose (1:1 v/v), which had been pre-swollen and previously washed three times in NETN (NET containing 0.5% Nonidet P-40). After incubation, the glutathione-agarose beads were washed once with NETN and twice with NET prior to elution of fusion proteins using NET containing 5 mM reduced glutathione. Alternatively, Laemmli sample buffer was added directly to the glutathione-agarose beads and boiled for 3-5 minutes in preparation for analysis by SDS-polyacrylamide gel electrophoresis (32).

Phosphorylation Reactions -

Purified fusion proteins (4 µg/assay) were incubated with purified $p34^{cdc2}$ in kinase buffer (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 µM ATP) containing [γ-^{32}P]ATP (30-60 µCi/nmol) in a total volume of 30 υL. Kinase reactions were initiated by the addition of purified $p34^{cdc2}$ (typically 50 pmolar units of enzyme) and the assay

performed at 30°C with constant agitation for the indicated length of time. Reactions were terminated by the addition of EDTA to a final concentration of 20 mM. Phosphorylated fusion proteins were immunoprecipitated by the addition of anti-$\alpha^{376-391}$ antiserum (0.5 υL/μg fusion protein) and protein A-Sepharose beads in antibody buffer (20mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS) supplemented with phosphatase inhibitors (20mM NaF, 20mM β-glycerophosphate). After incubation for 60 minutes on ice, the beads were collected by centrifugation and washed four times with L-buffer (phosphate buffered saline, pH 7.5, 1% Nonidet P-40, 0.5% deoxycholate) containing 1% aprotinin and phosphatase inhibitors. The washed beads were then resuspended in Laemmli sample buffer and the samples boiled for 3-5 minutes prior to analysis by SDS-polyacrylamide gel electrophoresis (32). Alternatively, fusion proteins were recovered from phosphorylation reactions by glutathione agarose bead purification as described in a previous section. Synthetic peptides (1 mM final concentration) were similarly phosphorylated with purified $p34^{cdc2}$ in kinase buffer (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 υM ATP) containing [γ-^{32}P]ATP (30-60 υCi/nmol) and allowing the reaction to proceed for up to 90 minutes at 30 °C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM. Phosphopeptides were

separated from the free [γ-^{32}P] using thin layer cellulose chromatography as described below using Scheidtmann buffer (33), and visualized with autoradiography.

To determine the stoichiometry of phosphorylation of fusion proteins, the following procedure was followed. Known amounts (2,4,6 υL) of each fusion protein were subjected to SDS-polyacrylamide gel electrophoresis, and visualized by staining with Coomassie Blue. Typically, each fusion protein displayed one major band corresponding to undegraded GST-fusion protein since it reacted on immunoblots with antibodies directed against the C terminus of CKII α (anti-$\alpha^{376-391}$) antibodies. Densitometry of the Coomassie Blue-stained gel was performed to determine the proportion of intact GST fusion protein. The phosphate incorporation into the undegraded GST fusion proteins were subsequently determined by analysis on a Phosphoimager (Molecular Dynamics). Stoichiometry of phosphorylation was calculated by the following formula: pmol of phosphate incorporated into intact fusion protein/pmol of intact fusion protein.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis -

The ^{32}P-labeled fusion proteins were visualized by autoradiography of unfixed dried SDS-polyacrylamide gels and

excised. Proteins were recovered from homogenized gel slices and oxidized with performic acid as previously described (34-36). The oxidized proteins were then resuspended in 25 υL of 50 mM NH_4HCO_3 and digested overnight (16 hours) at 56 °C with 10 υg of thermolysin in the presence of 1 mM $Cacl_2$. An additional 5 υg of thermolysin was then added and digestion continued for at least another 4 hours. Following digestion, samples were repeatedly lyophilized (four times) using a Speedvac concentrator. Thermolytic digests of the phosphorylated fusion proteins were subjected to electrophoresis using pH 1.9 buffer (15% acetic acid, 5% formic acid) followed by ascending chromatography with Scheidtmann buffer (isobutyric acid:pyridine:acetic acid:butanol:water (65:5:3:2:29)) as previously described (21,33). Phosphopeptides were visualized by autoradiography. Phosphoamino acid analysis of peptides was performed following partial acid hydrolysis as previously described (36). Briefly, individual spots were scraped from cellulose plates, and phosphopeptides were eluted from the cellulose with pH 1.9 buffer. Following removal of the buffer using a SpeedVac, phosphopeptides were subjected to partial acid hydrolysis, and partial hydrolysis products were subjected to two-dimensional electrophoresis as described previously (36).

Other methods-

Bovine CKII was purified (37) and phosphorylated using purified $p34^{cdc2}$ as previously described (20,21). Protein determinations were by the method of Bradford (38) using bovine serum albumin as standard. Autoradiography was performed using Kodak X-Omat film.

RESULTS AND DISCUSSION

Previous work had shown that sites within the C-terminal domain of the α subunit of CKII were phosphorylated in cells arrested in mitosis with nocodazole and that immunopurified $p34^{cdc2}$ was capable of phosphorylating the same sites *in vitro* (21). To provide additional evidence that the $p34^{cdc2}$ phosphorylation sites are localized to the C-terminal domain of CKII α, a GST fusion protein encoding the C-terminal 126 amino acids of CKIIa (GST-αC126) was tested as a substrate for purified $p34^{cdc2}$. As shown in Fig.3.1, GST-α 126 is effectively phosphorylated by $p34^{cdc2}$. By comparison, neither GST nor a GST fusion protein encoding the C-terminal 51 amino acids of CKII α' (GST-α'C51) is phosphorylated. In addition, no phosphorylation of GST-α C126 was observed when $p34^{cdc2}$ was omitted from the reaction mixture.

To determine whether GST-α C126 and intact CKII α are phosphorylated at the same sites, we performed comparative phosphopeptide mapping. Thermolytic digestion of phosphorylated GST-α C126 and purified CKII α resulted in the production of 4 major phosphopeptides that comigrated when phosphopeptides from each of the two samples were mixed

(Fig. 3.2). It is therefore apparent that all of the sites that are phosphorylated on CKII α by $p34^{cdc2}$ are present in the C-terminal 126 amino acids of the protein. Furthermore, since comparative phosphopeptide mapping experiments had previously demonstrated that phosphopeptides 1,2 and 3 obtained following *in vitro* phosphorylation of purified CKII comigrate with phosphopeptides obtained following ^{32}P-labelling of mitotic Jurkat cells (21), these results indicate that GST-α C126 is phosphorylated at sites that are phosphorylated in mitotic cells. We previously noted that purified CKII is phosphorylated at a site, represented by phosphopeptide d (Fig. 3.2), that was not detected from samples of CKII obtained from intact cells (21). It is apparent that this phosphopeptide, which is also present following phosphorylation of GST-α C126, does not represent an *in vivo* phosphorylation site.

Inspection of the amino acid sequence of the carboxyl terminal domain of CKII α revealed the presence of four residues, Thr-344, Thr-360, Ser-362 and Ser-370, that conform to the minimal consensus sequence for $p34^{cdc2}$ phosphorylation (39). Synthetic peptides corresponding to portions of CKII α containing each of the putative phosphorylation sites were synthesized and phosphorylated *in vitro* with purified $p34^{cdc2}$(see Table 3.1). It is interesting

to note that replacement of Ser-362 with alanine (compare peptide 3 with peptide 2) abolishes serine phosphorylation, suggesting that $p34^{cdc2}$ does not likely phosphorylate Ser-356 or Ser-357 within this peptide. In a similar vein, peptide 1 is exclusively threonine phosphorylated, demonstrating that $p34^{cdc2}$ does not likely phosphorylate Ser-343, Ser-348, or Ser-349 within the peptide.

To identify the $p34^{cdc2}$ phosphorylation sites on CKII α, a mutagenesis strategy was employed. We prepared fusion proteins in which one or more of the putative $p34^{cdc2}$ phosphorylation sites had been mutated to non-phosphorylatable alanine residues (see Table 3.2 for summary). Each of these fusion proteins was tested as an *in vitro* substrate for $p34^{cdc2}$ and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (see Fig. 3.3). Each of the fusion proteins displays at least one phosphorylated band with noticeably reduced electrophoretic mobility, except for the fusion protein in which all four putative phosphorylation sites had been mutated (Fig. 3.3K). This fusion protein yields a single phosphorylation band of unaltered electrophoretic mobility. In addition to differences in the locations or shift in electrophoretic mobility of each protein, differences in the extent of phosphorylation were observed for each fusion protein. Interestingly, fusion proteins containing both residues Thr-344 and Ser-370 (*lanes* A, B, C and I; TTSS, TTAS, TASS, and

TAAS, respectively) exhibited the most significant shifts in electrophoretic mobility and also produced some of the most intense bands. Taken together, these results suggest that Thr-344 and Ser-370 are important for the optimal phosphorylation of the C-terminal domain of CKII α by $p34^{cdc2}$. Phosphorylation of the wild-type GST-α C126 fusion protein achieved an approximate stoichiometry of 3 mol of phosphate/mol of protein (Fig. 3.4), while the AAAA mutant was phosphorylated to a stoichiometry of approximately 0.3 mol of phosphate/mol of protein.

Alterations in the intensity of phosphorylation and in the extent of the electrophoretic mobility shift of individual GST fusion proteins (GST-α C126 and corresponding mutants) suggested that some of the $p34^{cdc2}$ sites had been eliminated by mutation. To directly examine the phosphorylation pattern of the individual mutants, two dimensional phosphopeptide mapping procedures were utilized (Fig. 3.5). In all cases, the most highly phosphorylated form (*i.e.* the uppermost phosphorylated band observed in Fig. 3.3) was subjected to analysis. A number of observations are apparent from examination of these phosphopeptide maps. As evidenced by examination of maps D,F,G,J,K and L (ATSS, ATSA, AASS, AASA, AAAA and AAAS, respectively) mutation of Thr-344 to alanine results in loss of phosphopeptide a, indicating that this phosphopeptide

contains Thr-344. Phosphoamino acid analysis demonstated that phosphopeptide a contains exclusively phosphothreonine (data not shown) supporting this interpretation. Phosphopeptide b is absent on all phosphopeptide maps obtained from fusion proteins that are mutated at both Ser-362 and Ser-370 (*panels* H and K; TTAA and AAAA, respectively) suggesting that phosphopeptide b is derived from similar, if not identical, peptides that are phosphorylated at either Ser-362 or Ser-370. Phosphoamino acid analysis of phosphopeptide b shows that this peptide is composed exclusively of phosphoserine (data not shown).

The region of CKII α that contains the putative $p34^{cdc2}$ phosphorylation sites does not contain any charged amino acids (28). As a result, mono-phosphorylated peptides would be nearly neutral at pH 1.9 (36). Phosphopeptides containing more than one phosphate would have sufficient negative charge at pH 1.9 to migrate towards the positive electrode (to the left in Fig. 3.5). The minimal migration exhibited by phosphopeptides a and b in the electrophoretic dimension is consistent with the presence of only a single phosphate on each of these peptides. Phosphopeptide c, observed only in A (TTSS), exhibits the electrophoretic mobility of a peptide with a significant negative charge. Furthermore, this peptide contains both phosphothreonine and phosphoserine suggesting that it is indeed a multiply phosphorylated peptide (data not shown). When Ser-362 (*map*

B, TTAS) or Thr-360 (*map* C, TASS) were mutated to alanine residues, phosphopeptide c was not observed. Instead, phosphopeptide e was observed on *map* B (TTAS) and phosphopeptide f was observed on Map C (TASS). Mixing experiments indicated that both phosphopeptides e and f were less negatively charged than phosphopeptide c (data not shown). These results indicate that phosphopeptides e and f are phosphorylated at more than one site, but that they are phosphorylated to a lesser extent than phosphopeptide c. Furthermore, phosphoamino acid analysis indicated that spot f was phosphorylated exclusively on serine, whereas spot e contained a mixture of phosphoserine and phosphothreonine. These results suggest that the negatively-charged phosphopeptide c is a triply phosphorylated peptide that has been phosphorylated at Thr-360, Ser-362, and Ser-370. Mutation of Thr-360 to alanine (*map* C, TASS) results in the disappearance of phosphopeptide c, and a concomitant appearance of phosphopeptide f. Phosphopeptide f, which is exclusively serine phosphorylated, has a lesser negative charge than phosphopeptide c, but still behaves as a multiply phosphorylated peptide which is presumably phosphorylated at Ser-362 and Ser-370. Similarly, mutation of Ser-362 to alanine (*map* B, TTAS) results in the loss of phosphopeptide c with the gain of the less negatively charged phosphopeptide e, which is most likely phosphorylated at both Thr-360 and Ser-370. The presence of

phosphoserine and phosphothreonine in phosphopeptide e supports this conclusion. If phosphopeptides e and f are indeed diphosphorylated peptides, it would naturally follow that phosphopeptide c is a triphosphorylated peptide that is phosphorylated at Thr-360, Ser-362 and Ser-370. The greater negative charge and diminished chromatographic migration of phosphopeptide c in comparison to phosphopeptides e or f is consistent with the presence of an additional phosphate on the former peptide (36).

Phosphopeptide d, which is only observed on maps derived from CKII or GST fusion proteins that are phosphorylated *in vitro*, is present on all phosphopeptide maps, including the fusion protein in which all putative $p34^{cdc2}$ phosphorylation sites, Thr-344, Thr-360, Ser-362 and Ser-370 have been mutated to non-phosphorylatable alanine residues. In fusion proteins with alanine instead of Thr-360 (*maps* C,G,I,J,K,L), it was noticed that spot d (denoted as d' on maps) only contained phosphoserine (data not shown). By comparison, when threonine was present at residue 360, phosphoserine and phosphothreonine were detected (data not shown) at spot d (designated as spot d on the maps). This result suggests that spot d, which is not observed following the phosphorylation of CKII in cells (21), was a mixture of co-migrating or identical mono-phosphorylated peptides arising either from phosphorylation of Thr-360 or phosphorylation of an unidentified serine. The result that the 4 site mutant

(ie. AAAA) was phosphorylated by $p34^{cdc2}$ was somewhat unexpected since the most likely $p34^{cdc2}$ phosphorylation sites had been eliminated through mutagenesis. It would therefore appear that the additional phosphorylation site does not conform to the minimal consensus for $p34^{cdc2}$ phosphorylation since the C-terminal region of CKII α does not contain any additional serine residues that are followed by a proline with the exception of Ser-295.

Elimination of this site by expressing a GST fusion protein encoding residues 300-391 of CKII α did not abolish the ability of $p34^{cdc2}$ to phosphorylate the fusion protein (data not shown), suggesting that Ser-295 is not the unknown phosphorylation site. The phosphorylation of non-consensus residues by $p34^{cdc2}$ has been previously noted (39, 40). It is important to emphasize that phosphopeptide d is not observed on phosphopetide maps derived from CKII α that had been arrested in mitosis.

Overall, the results obtained by phosphorylation of mutant fusion proteins and resultant phosphopeptide maps support the conclusion that the preferred sites of phosphorylation by $p34^{cdc2}$ in cells are Thr-344, Thr-360, Ser-362 and Ser-370 on the CKII α subunit (summarized in Table 3.3). In fact, mutation of each of these residues to alanine results in elimination of all phosphopeptides that are detected following the phosphorylation of CKII α in mitotic

cells. Since $p34^{cdc2}$ also phosphorylates Ser-209 on CKII β in mitotic cells, the present results indicate that $p34^{cdc2}$ could phosphorylate the CKII holoenzyme to a stoichiometry of up to 10 moles phosphate/mole of $\alpha_2\beta_2$ tetramer in mitotic cells. The high stoichiometry of phosphorylation suggests that phosphorylation could regulate functional properties of CKII and that it could in some way participate in the burst of phosphorylation that accompanies the activation of $p34^{cdc2}$ at the G_2/M transition (1-4, 41-43).

At present, the role of phosphorylation in regulating the functions of CKII in mitotic cells remains speculative. There have been a number of studies in amphibian (44-46) or starfish oocytes (47) and in mammalian cells (48) that have suggested that the activity of CKII is regulated at different stages in the cell cycle. However, a direct link between these changes in the catalytic activity of CKII and the phosphorylation state of CKII has not been demonstrated. *In vitro* studies by Mulner-Lorillon et al. (19) demonstrated that CKII isolated from *Xenopus laevis* can be phosphorylated and activated by $p34^{cdc2}$. By comparison, when we examined the activity of CKII that had been isolated in its fully phosphorylated state by immunoprecipitation from mitotic cells, the catalytic properties of the enzyme were not significantly different from the unphosphorylated enzyme (21). The latter results suggest that phosphorylation of

CKII in mammalian or avian cells may not have direct effects on the enzymatic properties of CKII. Despite the lack of evidence that directly links the phosphorylation of CKII to changes in its catalytic activity during mitosis, there are suggestions that the functions of CKII could be altered during mitosis. For example, independent studies using immunofluorescence have demonstrated that CKII is associated with the mitotic spindle in dividing cells (49,50). The factors that control the interaction of CKII with the mitotic spindle remain uncharacterized. However, it should be noted that alterations in the ability of CKII to phosphorylate substrate proteins could be mediated by affects on its intracellular distribution or on its interaction with specific substrates without obvious effects on its enzymatic activity. In this regard, there are indications from the studies of Cardenas et al., (51) that the phosphorylation of topoisomerase II is increased in mitosis and that the major mitotic phosphorylation sites are CKII sites. At the restrictive temperature, in yeast harboring a temperature-sensitive form of CKII, topoisomerase II is hypophosphorylated, and the cells fail to divide. In mammalian cells, the phosphorylation of topoisomerase II is also elevated in mitosis (52). With the exception of topoisomerase II, very little is known regarding the stage in the cell cycle when CKII phosphorylates its substrates. It will certainly be of

interest to examine the phosphorylation of other CKII substrates to determine if any of these proteins are phosphorylated at the G_2/M transition. It is also noteworthy that the mitotic phosphorylation sites that have been identified on the α subunit of human CKII are highly conserved between mammalian species and are even present in the α subunit of chicken CKII (28,53). Interestingly, none of these sites are present on the α' subunit of CKII from either species. The latter observation suggests that the regulation, and perhaps other functional properties, of the α and α' isozymic forms of CKII could be distinct. Identification of the mitotic phosphorylation sites on the α subunit of CKII will undoubtedly facilitate efforts to define the role of CKII and its phosphorylation during cell division.

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Table 3.1 **Phosphoamino Acid Analysis of Phosphorylated Synthetic Peptides**

Peptide	Residues[a]	Synthetic peptide sequence[b]	Residue phosphorylated	Phosphorylation rate[c] (pmol/min/ul)
1	340-349	P-G-G-S-T-P-V-S-S-A	T	2.9
2	355-369	I-S-S-V-P-T-P-S-P-L-G-P-L-A-G	S,T	4.8
3[d]	355-369	I-S-S-V-P-T-P-A-P-L-G-P-L-A-G	T	5.6
4	364-375	L-G-P-L-A-G-S-P-V-I-A-A	S	4.3

[a]Amino acid numbering of human CKII α (as per ref. 28)
[b]Residues in bold are the residues that conform to the minimal consensus for $p34^{cdc2}$ phosphorylation
[c]Synthetic peptides were assayed at a concentration of 1mM using purified $p34^{cdc2}$ with an activity of 52 pmol/min/µl with 1mM Ser209 peptide as substrate.
[d]Residue underlined in peptide 3 represents a serine to alanine change

Table 3.2 **Phosphorylation Site Mutants of GST-αC126**

Designation	Sequence(CKIIα Residues 344-371)[a]	Mutations
TTSS	TPVSSANMMSGISSVPTPSPLGPLAGSP	none (wild-type)
TTAS	**********A*********	S^{362}/A
TASS	**********A**********	T^{360}/A
ATSS	A***************************	T^{344}/A
TTSA	***************************A*	S^{370}/A
ATSA	A**************************A*	$T^{344}/A;S^{370}/A$
AASS	A**********A**********	$T^{344}/A;T^{360}/A$
TTAA	**********A*******A*	$S^{362}/A;S^{370}/A$
TAAS	**********A*A*********	$T^{360}/A;S^{362}/A$
AASA	A**********A********A*	$T^{344}/A;T^{360}/A;S^{370}/A$
AAAS	A**********A*A*********	$T^{344}/A;T^{360}/A;S^{362}/A$
AAAA	A**********A*A*******A*	$T^{344}/A;T^{360}/A;S^{362}/A;S^{370}/A$

[a]Asterisks signify residues identical to the wild type sequence of human CKII α (ref. 28)

Table 3.3 **Summary of Phosphopeptide Mapping Data**

Phosphopeptide	Phosphoamino Acid	Probable Residue	Observed *In Vivo*[a]
a	Thr	Thr-344	Yes
b	Ser	Ser-362 or Ser-370	Yes
c	Ser, Thr	Thr-360 and Ser-362 and Ser-370	Yes
d	Ser, Thr	Thr-360, unknown Ser	No
d'	Ser	unknown Ser	No
e	Ser, Thr	Thr-360 and Ser-370	No
f	Ser	Ser-362 and Ser-370	No

[a]Phosphopeptides obtained following isolation of CKII α from ^{32}P labelled cells arrested in mitosis (ref. 21).

Fig 3.1 **Phosphorylation of GST Fusion Proteins**. A) Schematic representation of glutathione S-transferase (GST), and the GST-αC126 and GST-α'C51 proteins. GST-αC126 is composed of GST fused to the 126 carboxy-terminal amino acids of the α subunit of CKII (indicated by solid black rectangle). GST-α'C51 encodes GST and the 51 carboxy-terminal residues of CKII α' (indicated by striped rectangle). B) Equal amounts (4 υg) of purified GST (lanes 1 and 2), GST-αC126 (lanes 3 amd 4), and GST-α'C51 (lanes 5 and 6)proteins were assayed for phosphorylation in the presence (lanes 1,3 and 6) or absence (lanes 2,4 and 5) of purified $p34^{cdc2}$ using the *in vitro* phosphorylation conditions described in "Experimental Procedures". The proteins were recovered from the phosphorylation reactions using glutathione agarose before analysis on a 12% SDS-polyacrylamide gel, and visualization by autoradiography.

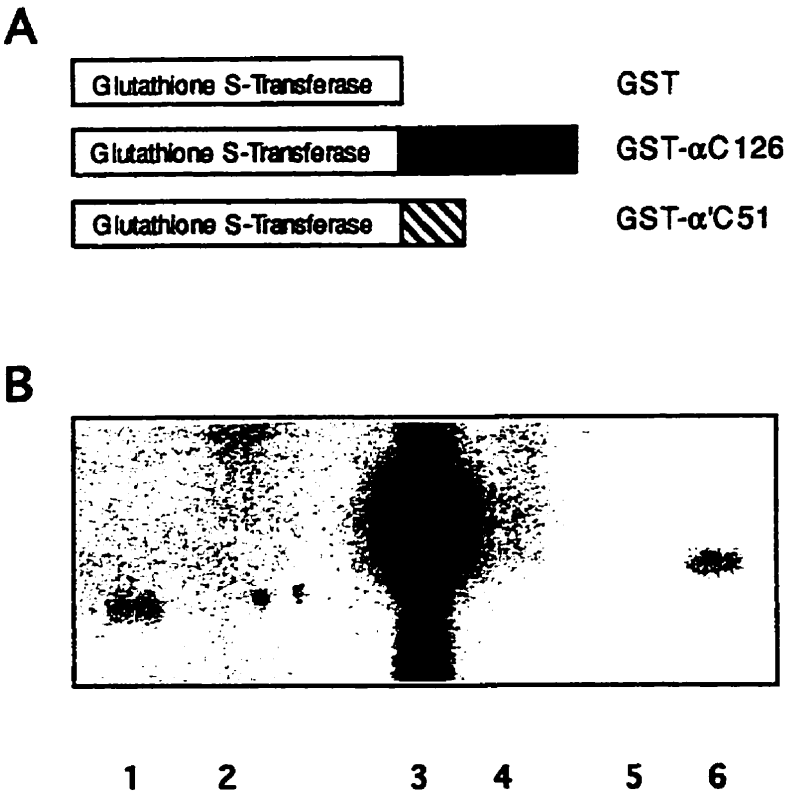

A
Glutathione S-Transferase
GST
Glutathione S-Transferase
GST-αC126
Glutathione S-Transferase
GST-α'C51
B
1
2
3
4
5
6

Fig. 3.2 **Comparative Phosphopeptide maps of GST-αC126 and of the α subunit of CKII phosphorylated *in vitro* by purified p34^{cdc2}.** GST-αC126 and purified bovine CKII were phosphorylated *in vitro* using purified p34^{cdc2} and were then immunoprecipitated from kinase reactions using anti-$\alpha^{376-391}$ antiserum. Immunoprecipitates were analyzed by autoradiography after separation of proteins on a 12% SDS-polyacrylamide gel. The phosphorylated fusion protein and the α subunit of CKII were recovered from homogenized gel slices excised from the SDS-polyacrylamide gel. The samples were exhaustively digested with thermolysin and then separated by electrophoresis at pH 1.9 (horizontal dimension with anode to the left), followed by ascending chromatography as described in "Experimental Procedures". The "MIX" phosphopetide map was obtained by mixing aliquots (equal cpm) of each phosphorylated sample (GST-αC126 and the α subunit of CKII) prior to two-dimensional separation. The positions of the origins are marked by arrows and the letter O. Individual phosphopeptides are identified with letters as in Ref. 21.

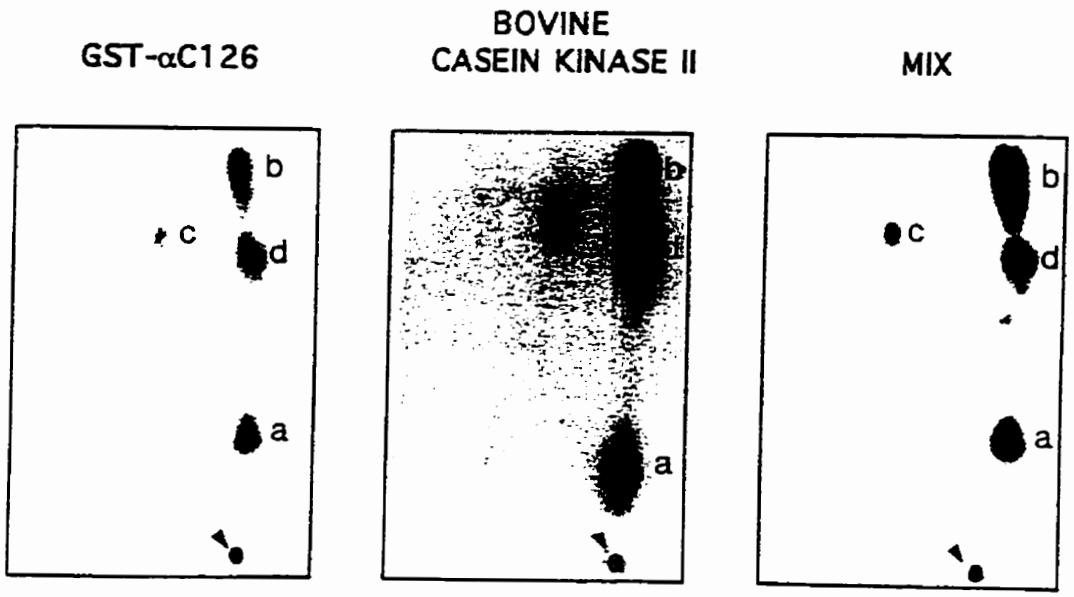

GST-αC126
BOVINE
CASEIN KINASE II
MIX
b
c
d
a
b
a
b
c
d
a

Fig 3.3 **Phosphorylation of GST-αC126 fusion proteins by p34^{cdc2}**. Wild-type and mutant GST-αC126 fusion proteins were phosphorylated *in vitro* for 90 min. using purified p34^{cdc2} as described under "Experimental Procedures". Equivalent amounts of each fusion protein were subsequently subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel, and phosphoproteins were visualized by autoradiography. Lanes A-L contain the following GST fusion proteins, respectively: TTSS, (i.e. wild-type GST-αC126), TTAS, TASS, ATSS, TTSA, ATSA, AASS, TTAA, TAAS, AASA, AAAA, AAAS. Fusion protein designation is according to Table 3.2 and represents the identity of the amino acid residues present at positions 344, 360, 362, and 370, respectively (numbering according to the deduced sequence of human CKII α; see ref. 28).

A B C D E F G H I J K L

Fig. 3.4 **Time course of phosphorylation of wild-type GST-αC126 (TTSS) and four-site GST-αC126 mutant (AAAA)**. Equal amounts (4 υg) of each fusion protein were phosphorylated *in vitro* using purified $p34^{cdc2}$. Aliquots of the wild-type GST-αC126 fusion protein (TTSS) (open squares), or of the four-site GST-αC126 mutant (AAAA) (open circles) assay mixture were taken at the various time points indicated, and phosphate incorporation was determined as described under "Experimental Procedures".

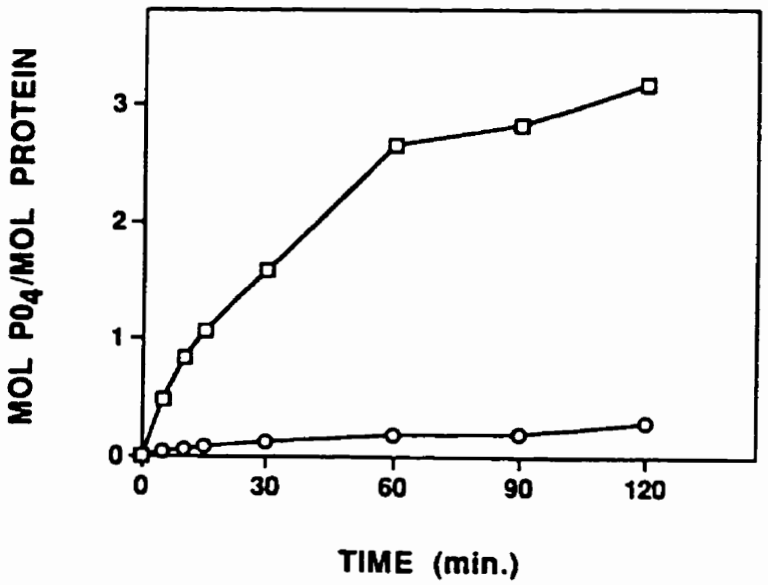
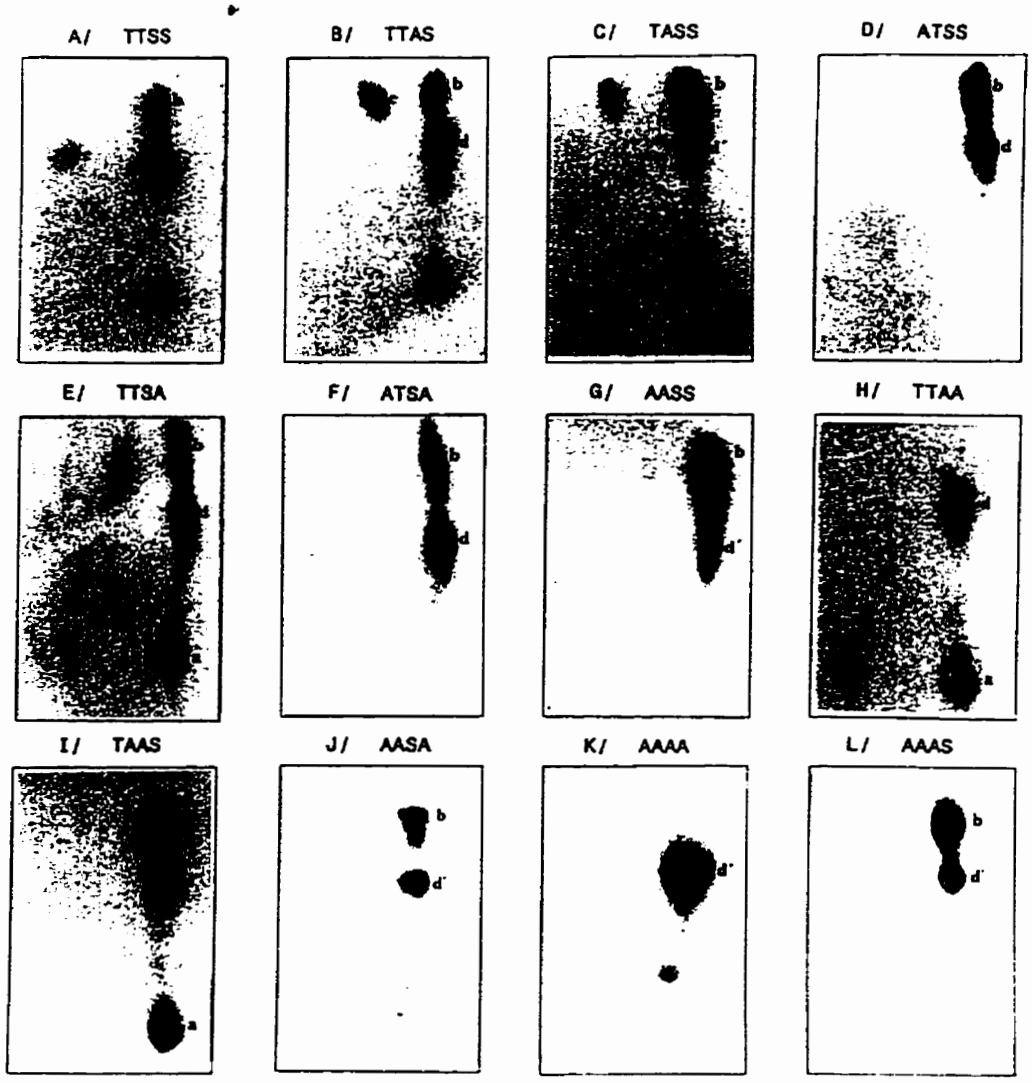

MOL PO_4/MOL PROTEIN
3
2
1
0
0
30
60
90
120
TIME (min.)

Fig. 3.5 **Thermolytic phosphopeptide mapping of GST-αC126 fusion proteins**. Fusion proteins containing different forms of the carboxyl terminal domain of the a subunit of CKII were phosphorylated *in vitro* using purified $p34^{cdc2}$ followed by immunoprecipitation using anti-$\alpha^{376-391}$ antibodies as described in the legend to Fig. 3.4. Phosphorylated fusion proteins were recovered from SDS-polyacrylamide gel slices, oxidized with performic acid, and exhaustively digested with thermolysin. Thermolytic peptides were then separated in two dimensions as described in the legend to Fig. 3.3 Phosphopeptide maps were visualized by autoradiography. Letters over each map represent the identity of the amino acid residues present at positions 344, 360, 362, and 370 respectively (see Table 3.2). Individual phosphopeptides are numbered.


A/ TTSS
B/ TTAS
C/ TASS
D/ ATSS
E/ TTSA
F/ ATSA
G/ AASS
H/ TTAA
I/ TAAS
J/ AASA
K/ AAAA
L/ AAAS

CHAPTER 4

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL PROTEIN THAT INTERACTS WITH PROTEIN KINASE CK2; EVIDENCE FOR ISOFORM-SPECIFIC INTERCATIONS WITH CK2.

Preface -

CK2 is involved in a myriad of cellular processes throughout the cell, however its cellular regulation is poorly understood. With the observations that the CTDs of CK2α and CK2α' are highly conserved in higher eukaryotes, do not influence catalytic activity, and do not appear to influence the formation of the CK2 holoenzyme, coupled with the observation that CK2α contains a "P-X-X-P" motif, which is a motif that has been shown to direct protein:protein interaction in other proteins, led us to hypothesize that the CTDs of CK2α and CK2α' may contribute to the regulation of CK2 through protein:protein interactions.

With the emerging identification of scaffold, anchoring and docking proteins that can interact and regulate the time and cellular locations at which certain kinases are activated (81,82,83), a similar form of regulation through specific protein associations may exist for CK2. In fact, it

has been demonstrated that interaction of CK2 with proteins such as CD5 and $p21^{waf1/cip1}$ can affect CK2 activity (31,33). In this regard, I describe how our laboratory used the yeast two-hybrid system to identify a novel protein which displays isoform interaction specificity for CK2α, but not CK2α'.

Contribution by the first author -

I performed all of the experiments described in this manuscript, except for the isolation of the Phu clone from the yeast, a portion of the reconstruction experiments, and the immunoblot analysis of anti-Phu immunoprecipitation experiment. I prepared most of the figures, and wrote the entire manuscript under the editorial supervision of David Litchfield and Dan Gietz.

Identification and characterization of a novel protein that interacts with protein kinase CK2; evidence for isoform-specific interactions with CK2.

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Short running title: Identification of a novel CK2 interacting protein.

ABSTRACT

The catalytic subunits of protein kinase CK2 (CK2), CK2α and CK2α', are closely related to each other, but differ completely at their unique C-terminal domains (CTDs). Reported differences between CK2α and CK2α', coupled with the observation that there is a "P-X-X-P" motif located within the unique CTD of CK2α, suggest that functional differences may exist between CK2α and CK2α', and that these differences could be regulated by the ability of CK2α or CK2α' to interact with specific protein partners in cells.

In order to examine this possibility we used the yeast two-hybrid system to screen a EBV-transformed human B-cell library in order to identify potential interaction partners for CK2α or CK2α'. We report the identification and characterization of a novel protein, designated as Phu. We observed that Phu is able to interact with CK2α, but not with with CK2α', in a yeast two-hybrid system, and in co-immunoprecipitation experiments with HA epitope tagged CK2 fusion proteins, and Enhanced Green Fluorescent Protein (EGFP)-Phu fusion protein in Saos-2 cell lysates. Moreover, we observed a redistribution of the prominently nuclear fluorescence of EGFP, to a cytoplasmic/plasma membrane fluorescence of EGFP-Phu, in Cos 7 cells. Using antibodies directed against the carboxy-terminal-domain of Phu, we show that Phu runs as a protein of 50 kD in denaturing SDS-

polyacrylamide gels, and that we can immunoprecipate CK2 activity as determined using the CK2 specific synthetic peptide "RRRDDDSDDD". Of note, is the identification of a putative pleckstrin-homology (PH) domain within the N-terminal deduced amino acid sequence of Phu. These findings suggest that Phu can localize to the plasma membrane, and potentially sequester CK2α in the cytoplasm and/or the plasma membrane.

INTRODUCTION

Protein kinase CK2 (CK2) is an essential, highly conserved, protein serine/threonine kinase present in all eukaryotic cells (reviewed in 1-6). CK2 has been reported to phosphorylate a broad range of cellular proteins located in a variety of cellular compartments (mainly the nucleus and cytoplasm), and is involved in important cellular processes such as transcription, translation, morphogenesis and cell cycle progression (reviewed 1-7). These observations support an important role for CK2 in a variety of cellular functions, however its specific role and mode of regulation in cells remain poorly understood. CK2 activity is not responsive to any known second-messengers, and appears to be, to some extent, constitutively active in cells (reviewed 4-7).

CK2 is a tetrameric protein comprised of two regulatory subunits (CK2ß) and two catalytic subunits (CK2α and/or CK2α'). CK2α and CK2α' are the products of separate genes, and their amino acid sequences are highly conserved between higher eukaryotes (reviewed in 7). In fact, in mammals and birds, CK2α and CK2α' exhibit greater than 90% identity over their 330 N-terminal amino acids (reviewed in 7). This N-terminal sequence identity is in stark contrast to the unrelated C-terminal sequences of CK2α and CK2α' which are completely unrelated (8,9,10). The sequence divergence

between the C-terminal domains (CTDs) of CK2α and CK2α' suggests that important functional differences which may exist between the two different catalytic isozymes result from these unique sequences.

Previous studies have failed to demonstrate significant catalytic differences between CK2α and CK2α' *in vitro* (11). These results likely reflect the high similarity of the catalytic domains between CK2α and CK2α'. However, differences between CK2α and CK2α' have been reported in cells. For example, in HeLa cells, differences in the localization of CK2α and CK2α' have been observed, with a further suggestion that the localization of both CK2α and CK2α' may be altered in a cell cycle dependent manner (12). However, these differences in subcellular localization may be cell type specific, since major differences in the localization of CK2α and CK2α' have not been observed in all cells where they have been examined (13,14). It has also been demonstrated that CK2α, but not CK2α', is phosphorylated in mitotic cells of mammalian and avian origin (15), suggesting that the functions of the two isoforms are independently regulated during cell division. The mitotic sites of phosphorylation of CK2α were identified as T^{344}, T^{360}, S^{362}, and S^{370} , all of which are located within the CTD of CK2α (16). Interestingly, the presence of a " P-X-X-P " motif is observed adjacent to two of the phosphorylation sites. It is important to note that

" P-X-X-P " motifs have been implicated in protein:protein interaction with SH3 domains of a variety of regulatory proteins (17,18,19).

Biochemical studies have localized CK2 activity within the cytoplasm, the nucleus and with other cellular structures including the plasma membrane (1,2,20,21). Immunofluorescence studies have confirmed that CK2 is localized in the nucleus and in the cytoplasm (12,13,14,22,23). The factors or mechanisms that regulate the subcellular distribution of CK2 remain poorly understood. In this regard, there is mounting evidence demonstrating that CK2 interacts with a variety of cellular proteins. For example, CK2ß has been reported to interact with FGF-2 (24), A-Raf (25,26), Nopp140 (27), p53 (28,29,30), $p21^{WAF1/CIP1}$ (31), c-Mos (32) and CD5 (33). CK2α and CK2α', have also been observed to interact with specific protein partners. For instance, both CK2α and CK2α' have been shown to interact with nucleolin (34),and ATF1 (35,36), while CK2α has been shown to interact with HSP90 (37,38), and with PP2A (39). Intriguingly it was demonstrated that PP2A associated with free CK2α, but not with CK2α bound to CK2β (39). Furthermore, the domain of CK2α implicated in the interaction with PP2A was localized to the sequence H^{166}-E-H-R-K-L (human and chicken CK2α), which is one of the few regions of non-identity between the N-termini of CK2α and CK2α' (H-Q-Q-K-K-L). Collectively, these observations

suggest that the subcellular localization of CK2, and presumably its ability to phosphorylate a number of its target proteins, may be regulated by interactions with specific protein partners. The resulting compartmentalization of CK2 could provide a mechanism for regulating CK2 activity in cells. In fact, this mode of regulation could reconcile the observations that although CK2 is a predominantly nuclear enzyme, it can also phosphorylate cytoplasmic proteins, as well as nuclear proteins.

The possible regulation of cellular CK2 function through its interactions with specific protein partners together with the observed differences between CK2α and CK2α' lead us to hypothesize that the catalytic subunits of CK2 are involved in isozyme-specific protein:protein interactions, and that these interactions may be mediated through the unique CTDs of CK2α or CK2α'. To examine this hypothesis, we used a yeast two-hybrid system to screen a EBV-transformed human B-cell library with full length CK2α or CK2α', as well as their respective CTDs as bait. In this study we report the isolation and identification of a novel protein which binds CK2α, but not CK2α'. Based on its localization to the plasma membrane, we propose that this novel protein may act to sequester active CK2 in the cytoplasm.

EXPERIMENTAL PROCEDURES

Materials -

Human osteosarcoma Saos-2 cells and SV40 large T transformed green monkey kidney Cos 7 cells were obtained from ATCC (Bethesda, Maryland) and were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Gibco) supplemented with 10% fetal calf serum (Gibco). Anti-HA antibodies (ie. 12CA5 monoclonal antibodies) were obtained from BABCO, and anti-GFP antibodies were obtained from Clontech or were a generous gift from Dr. L. Berthiaume (University of Alberta). Rabbit antibodies against CK2β were described previously (40). Nitrocellulose and PVDF for immunoblots as well as reagents for the colorimetric development of immunoblots were obtained from Biorad. Reagents for enhanced chemiluminescence (ECL) were obtained from Amersham. All other reagents were of reagent grade.

Methods -

Plasmid Constructs -

pGBT9 constructs. Full length cDNAs encoding CK2α or CK2α', were subcloned into the *Bam*HI site of the pGBT9 vector. Appropriate orientation was verified by DNA sequencing. To prepare constructs encoding the carboxy-terminal-domains (CTDs) of CK2α or CK2α', the cDNA encoding the respective CTDs were amplified by PCR with restriction

sites (*Eco*R1 and *Bam*H1) at either end to facilitate subcloning. A 217-basepair fragment representing the CK2α-CTD was amplified from an sk⁺/CK2α template using the following primers:

5' CCGGAATTCGCTCGAATGGGTTCATCT 3'(sense primer with *Eco*RI site)

5' CGGATCCACCTCTGCTCAGGCATC 3'(antisense primer with *Bam*HI site)

Similarly, a 190-basepair fragment containing the CTD of CK2α' was amplified from an sk⁺/CK2α' template using the following primers:

5' CCGGAATTCTCCCAGCCTTGTGCAGAC 3'(sense primer with *Eco*RI site)

5' GTAAAACGACGGCCAGT 3' (M13 -20 primer)

The amplified fragments were directly cloned into the pCRII vector (Invitrogen) according to the manufacturer's recommendations. The sequences of all amplified fragments were confirmed by sequencing the double-stranded pCRII vector with the 5' CATTTAGGTGACACTATAG 3' primer (Sp6 promoter) using a T7 sequencing kit (Pharmacia Biotech). The respective *Eco*RI /*Bam*H1 fragments were then subcloned into *Eco*RI and *Bam*HI digested pGBT9 vector.

To generate CK2α-CTD (4D), the nucleotides coding for the amino acid residues representing the four mitotic sites of phosphorylation at amino acid residues (T^{344}, T^{360}, S^{362}, and S^{370}) within CK2α (16), were mutated to replace each of the

phosphorylation sites with aspartic acid. These mutations were engineered in order to mimic the charges brought on by phosphorylation of the four CK2α residues. Using two rounds of PCR amplification, we produced the CK2α-CTD (4D) mutant. In the first round of PCR, two products were made using the following primers:

5' CCT GAG CTA CTT GTA GAC TA 3'(sense primer for the 1st product), 5' TGG CAC TGA AGA AAT CCC TGA CAT CAT ATT GGC GCT GCT GGG ATC ACT GCC CCC 3'(antisense primer for the 1st product that introduces the T^{344}/D mutation), 5' GGG ATT TCT TCA GTG CCA GAT CCT GAT CCC CTT GGA CCT GCA GGC GAT CCA GTG 3' (sense primer for the 2nd product that introduces T^{360}/D, S^{362}/D and S^{370}/D mutations), and 5' TCC CCC ACC TCT GCT CAG 3'(antisense primer for the 2nd product). After amplification, the DNA fragments of interest were gel purified, denatured, allowed to anneal and then subjected to a second round of PCR using the sense primer from the 1st product in the first round of PCR and the antisense primer from the 2nd product in the first round of PCR as primers. The PCR products from the second round of amplification were directly cloned into the pCRII vector and sequenced. An *Eco*RI/*Sph*I fragment from the resultant plasmid encoding the 4D variant of the CTD of CK2α was then used to replace a similar fragment in pGEX-CK2α-CTD (16). Using this construct as a template, *Eco*RI and *Bam*HI sites were generated with PCR using the following primers:

5' CCGGAATTCGCTCGAATGGGTTCATCT 3'(sense primer with *Eco*RI site)

5' CGGATCCACCTCTGCTCAGGCATC 3'(antisense primer with *Bam*HI site). The plasmid pGBT9-CK2α-CTD(4D) construct was derived from this construct using the strategy described above for pGBT9-CK2α-CTD and pGBT9-CK2α'-CTD. All constructs were sequenced to confirm identity and to ensure that no mutations were introduced during amplification.

pEGFP constructs. To generate constructs for the expression of green fluorescent fusion protein fusions of Phu, a 1.5 kb *Bgl*II fragment encoding the entire open reading frame encoded by the two hybrid positive was subcloned into the *Bgl*II site located within the multiple cloning site of the pEGFP-C2 vector (Clontech) to yield the pEGFP-C2-Phu construct. Alternatively the purified *Bgl*II fragment was further digested with *Bam*HI and the resulting 300 b.p. fragment encoding the C-terminal 102 amino acid residues of PHU was gel purified and then subcloned into the *Bam*HI site located within the multiple cloning site of the pEGFP-C2 vector to yield pEGFP-C2-Phu-CTD. Orientation for pEGFP-C2-Phu was confirmed by *Sac*I digest, while orientation for pEGFP-C2-Phu-CTD was confirmed by *Stu*I and *Bam*HI double digest.

Other constructs. A strategy similar to that described in the preceding paragraph was used to subclone the 1.5 kb *Bgl*II fragment and the 300 bp *Bam*H1/*Bgl*II fragment into

pGEX-3X to generate glutathione S-transferase (GST) fusion proteins encoding the entire open reading frame (designated GST-Phu) and a GST fusion protein encoding the C-terminal 102 residues of Phu (designated GST-Phu-CTD). A plasmid encoding a GST fusion protein of CK2α was constructed by subcloning the *Bam*H1 fragment encoding full length CK2α into the *Bam*H1 site of pGEX-3X.

Constructs encoding CK2 subunits with N-terminal HA epitope tags in pRC/CMV (Invitrogen) were previously described (14).

Anti-Phu Antibodies. Anti-Phu antibodies were raised in rabbits by BABCO (Richmond California) using GST-Phu-CTD as the antigen. To characterize these antibodies, lysates were prepared from human osteosarcoma Saos-2 cells using Laemmli sample buffer and were analysed on Western blots that were developed with pre- or post-immune serum from either of the two immunized rabbits in the presence or absence of GST-Phu-CTD or GST. Antiserum from either of the two immunized rabbits, but not the pre-immune serum, recognized a band of approximately 50 kD in extracts of Saos-2 cells that was blocked by the inclusion of GST-Phu-CTD, but not by GST, during the incubation with antibody.

Transformation and Maintenance of Yeast - The yeast strain PJ69- 4a [MATa, ade2, trp1Δ901, leu 2-3, 112,

his3Δ200, gal4Δ, gal80Δ, ura3-52, GAL7-lacZ::met1, GAL2-ADE2::ADE2, GAL1-HIS3::LYS2 (courtesy of Dr. Philip James, University of Wisconsin)] was transformed with the various pGBT9-CK2 constructs using the method of Schiestl and Gietz, (1989), (41). The transformants were selected on plates of synthetic complete medium minus Trp (Trp^-) after growth for 3-5 days at 30°C. Separate transformants containing each of the different pGBT9-CK2 constructs were further grown in liquid medium (Trp^-), then transformed with a MATCHMAKER cDNA library (Clontech) derived from EBV-transformed human lymphocytes (B cell population). Transformants were plated on synthetic complete medium minus Trp and minus Leu (Trp^- Leu^-), as well as on complete medium minus Trp, minus Leu, minus His, and minus Ade ($Trp^-Leu^-His^-Ade^-$) and grown for 3-6 days at 30°C. Colonies that grew under the latter conditions were selected for further characterization.

Screening of yeast-two-hybrid positives for CK2ß - Since we had previously demonstrated that interactions between CK2α (or CK2α′) and CK2β can be detected using the two hybrid system (42), we utilized a PCR strategy to examine positive colonies from the screens performed using CK2α or CK2α′ as the bait for the presence of the cDNA encoding CK2β. For this analysis, yeast were grown overnight at 30° C in liquid culture. Yeast were disrupted by vortexing with glass beads and plasmid DNA was isolated as

described previously (43). The primers used for PCR were: 5'GCGGGGATCCTGAGCAGCTCAGAGGAG 3'(sense primer located within the domain of CK2β that interacts with CK2α), and 5'CTACCAGAATTCGGCATGCCGGTAGAGGTGTGGTCA 3'(antisense primer located within the ADH-terminator of pACT). Colonies that were positive for CK2β were not further analysed.

Isolation of the Phu clone -

After excluding all of the positives that encoded CK2β, one positive from the screen with CK2α was further characterized. Plasmid DNA was obtained from yeast by glass bead preparation and electroporated into the KC8 strain of *Escherichia coli*. To select for those bacteria that harbored the pACT plasmid, bacteria were selected on M9/Leu$^-$ minimal media plates. Plasmid DNA was then isolated and used to re-test for interactions with Gal4 DNA binding domain fusions of CK2α or CK2α' in yeast, as described in a preceding section. Transformations were also performed with a number of control constructs (as indicated). Plasmid DNA was also used as a template for sequencing manually using T7 polymerase or by automated sequencer (Perkin Elmer-Applied Biosystems).

Northern Blot Analysis - A Multiple Tissue Northern blot was purchased from Clontech and probed as recommended in the accompanying manual. Probes that were labelled with ^{32}P were

as follows: an *Xho1* fragment of the Phu cDNA, or the full length cDNAs encoding CK2α, CK2α' or CK2β.

In Vitro binding Assay - A GST fusion protein encoding full length CK2α and GST itself were expressed in bacteria and purified using glutathione agarose as described previously (16). Purified GST-CK2α and GST were then coupled to Affigel-10 (Biorad) at a concentration of 4 mg/ml according to the manufacturer's recommendations. Radiolabelled (^{35}S-labelled) Phu was produced by *in vitro* transcription and translation using a "TnT" kit (Promega) with T7 polymerase according to manufacturer's recommendations. For this procedure, a PCR product encoding Phu was generated using the *Pfu* heat stable DNA polymerase (Stratagene) from pACT-Phu using the following primers: 5' TAA TAC GAC TCA CTA TAG GGA GAC CAC ATG GAT GAT GTA TAT AAC TAT CTA TTC (T7-GAL4 sense primer) and 5' CTA CCA GAA TTC GGC ATG CCG GTA GAG GTG TGG TCA 3' (pACT-ADH terminator antisense primer). Radiolabelled Phu (5 µL of the reticulocyte lysate reaction) was incubated for 1 hour at 4°C with 5 µL of Affigel-10-GST beads in a total volume of 50 µL in interaction buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 0.05% NP-40) supplemented with 0.1 mM PMSF and 1% aprotinin. After the 1 hour incubation, the Affigel beads were collected by centrifugation, the

supernatant was removed and the beads were washed 3 times with the interaction buffer. Proteins retained on the Affigel beads were eluted by the addition of Laemmli sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis. Radiolabelled proteins were visualized using a phosphorimager (Molecular Dynamics).

Transfection of Cos 7 AND Saos-2 cells - Cells were grown to 80% confluence, then split 1:8 into 100 x 20 mm culture plates or 35 x 20 mm wells containing coverslips. The following day, the media was changed 2-5 hours prior to transfection. Transfections were carried using calcium phosphate as previously described (44). Plasmids encoding various pEGFP constructs were added at concentrations of 5 µg per 100 x 20 mm culture plates, or 2 µg per 35 x 20 mm wells of a 6-well flat bottom tissue culture plate. The various pRc/CMV vectors encoding CK2 constructs were added at concentrations of 15-25 µg per 100 x 20 mm culture plate or 5-10 µg per 35 x 20 mm well of a 6-well flat bottom tissue culture plate. Precipitates of DNA were left on cells for 16-18 hours after which time, the cells were washed thorougly with PBS pH 7.4-7.5 to remove the precipitate. Media was added to cells which were incubated a further 24 hours. Cells were then washed thorougly with PBS and the liquid removed. At this time, cells grown on coverslips were

mounted onto glass slides for fluorescence microscopy, while cells on the plates were frozen at -80°C until needed.

Cell extracts were initially prepared by scraping cells from plates in lysis buffer (100mM Tris-Cl pH 7.5, 2mM EDTA, 100mM NaCl, 1% Triton-X-100, 10mM AEBSF, 1% Aprotonin, 2 μg/μL Leupeptin, 2 μg/μL Pepstatin A), followed by sonication (3 X 10 second bursts) on ice. The resulting lysates were centrifuged at 16 000 x g for 10 minutes. The supernatants were collected and used immediately for immunoprecipitations or alternatively stored at -80°C. Expression of relevant proteins was confirmed using Western blot analysis.

Immunoprecipitation - Immunoprecipitations were carried out in a 1:1 mixture comprised of transfected cell extract supernatants (described in previous section), with binding buffer (200mM NaCl, 20% glycerol, 1% Triton-X-100). Anti-HA immunoprecipitations were performed using a combination of anti-HA antibodies (1/250) and rabbit-anti-mouse IgG (1/20). Anti-Phu immunoprecipitations were performed using anti-Phu (1/250). Samples were incubated on ice, with gentle rocking, for 1 hour before addition of 20 μL of protein-A-sepharose. Samples were then allowed to incubate a further 30 minutes. Sepharose beads were washed with 3 x 500 μL of a 1:1 mixture of lysis buffer:binding buffer, then resuspended in Laemmli

sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. Proteins were detected using either anti-GFP antibody (1/2000), anti-Phu antibody (1/1000), or anti-HA (1/2000) as primary antibody and then appropriate secondary antibodies conjugated to horseradish peroxidase followed by detection with enhanced chemiluminescence.

Immunoprecipitations of endogenous Phu from non-transfected Saos-2 cells were performed by extracting Saos-2 cells with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS) (45) supplemented with protease inhibitors (10 μg/ml leupeptin, 0.1 mM PMSF, 1% aprotinin). These extracts were clarified by centrifugation as described above and incubated with anti-Phu antibodies (1/250) for 2 hours at 4°C. Since it was apparent that the anti-Phu antibodies recognized a protein in cell extracts of approximately 50 kD, antibodies were initially crosslinked to Protein A Sepharose using dimethylpimelimidate as described (46) to minimize interference from the immunoglobulin Heavy chain. Immunoprecipitates were washed 3 times with RIPA buffer and once with 20 mM Tris, pH 7.5 before analysis on Western blots using anti-Phu antibodies. For these experiments, blots were developed colorimetrically using BCIP and NBT as the substrates for alkaline-phosphatase conjugated secondary goat anti-rabbit antibodies.

Immune-complex kinase assays. Immunoprecipitations were performed as described in the preceding section with the exception that cells were lysed with 1% NP-40 in PBS instead of RIPA buffer. Immunoprecipitates were washed twice with 1% NP40 in PBS and then twice with 50 mM Tris, 150 mM NaCl. Assays to measure CK2 activity were performed for 10-20 minutes at 30°C with gentle shaking in a 30 µl reaction containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 20 µM ATP (specific activity approximately 0.01 µCi/pmol) using the synthetic peptide RRRDDDSDDD (0.1 mM) as a specific substrate for CK2 (47). Reactions were initiated by the addition of immunecomplex and were terminated by the addition of EDTA to a concentration of 25 mM. Reactions were centrifuged briefly to pellet immunecomplexes and half of the supernatant was spotted on P81 paper to measure incorporation of ^{32}P into synthetic peptide as previously described (47). All samples were assayed in the presence or absence of synthetic peptide to control for background phosphorylation of proteins in the immunecomplexes.

Visualization of EGFP fusion proteins in transfected

cells - Transfected Cos 7 cells were visualized using a "Zeiss Axiophor Photomicroscope". EGFP fluoresence was observed using a 510 filter (450-490/515-565), while DAPI was seen with a 395 filter (385/420). Images were captured using a "Photometrics Series 200-CCD camera system", and analyzed with "IPLab Spectrum P" Macintosh software.

RESULTS

Yeast two-hybrid screen - In order to identify protein binding partners of CK2α or CK2α', we performed independent yeast two-hybrid screens of a human B-cell cDNA library using constructs encoding full-length CK2α, and CK2α', as well as the respective CTDs (Fig. 4.1A). The CTDs of CK2α or CK2α' were each utilized in screens in order to examine the possibility that these unique CTDs were sufficient for interactions with binding partners. In the event that the CTDs were not sufficient for interactions, we also performed screens with full length CK2α and CK2α'. Screens with each of these constructs were performed simultaneously.

As summarized in Fig. 4.1B, neither of the CTD constructs yielded any positive clones. However, using the full length CK2α and CK2α' we obtained 243 and 196 positives, respectively. We expected that a number of the positives could be CK2β, the regulatory subunit of CK2 that is known to interact strongly with both CK2α and CK2α'in yeast two hybrid assays (42). Therefore, we used a PCR screening procedure to test positive colonies for the presence of the CK2β cDNA being expressed as a fusion with the transcriptional activation of Gal4. The results from the PCR analysis demonstrated that the majority of the positives contained CK2ß a result which validates the utility of the yeast two hybrid system for the identification of binding

partners for the catalytic subunits of CK2. A single yeast transformant, designated #38, did not show the presence of the CK2β cDNA. Consequently, the pACT library plasmid was isolated and analyzed further.

Specificity of interaction of pACT-38 with CK2α - In order to determine whether the protein encoded by the pACT plasmid in colony #38 (designated pACT-38) encoded a protein which specifically interacts with CK2α, we examined its ability to interact with CK2α as well as other proteins in the yeast two-hybrid assay (Fig. 4.1, panel C). Interactions were only detected when pACT-38 was co-expressed with CK2α. Despite the high degree of similarity between CK2α and CK2α', we did not observe any interaction with CK2α'. This latter result is consistent with the absence of this cDNA arising from the screen that was performed with CK2α'. In these reconstruction assays, the integrity of the CK2α and CK2α' constructs was confirmed by testing them successfully for interactions with pACT-CK2ß (data not shown). The interaction of the pACT-38 encoded protein with CK2α, but not with CK2α', suggests that differences existing between CK2α and CK2α' are sufficient to confer an interaction specificity. Moreover, the observation that the protein encoded by pACT-38, which we have called Phu, did not interact with either the CK2α-CTD or CK2α'-CTD, indicates that the CTDs by themselves are not sufficient to mediate the interaction between CK2α and Phu.

The ability of Phu to interact directly with CK2α was tested *in vitro*. In fig. 4.2, panel A, we detected a major radiolabelled translation product of approximately 50 kDa in the lysate containing the pACT-38 DNA (lane P), which was not present in the control lysate (lane C). Using GST-CK2α and GST proteins as affinity ligands in pull-down assays, it was evident that the Phu protein interacts with GST-CK2α, but not with GST (Fig. 4.2, panel B). Quantitation of the amount of radioactivity that was recovered on affigel beads (Fig. 4.2B) or remained in the supernatant (Fig. 4.2C) indicated that approximately 50% of the Phu was retained on the GST-CK2α, whereas the amount of Phu that was retained on GST alone was negligible. In addition, Phu exhibited negligible interactions with GST-CK2β (data not shown). Taken together, the yeast two hybrid-system results, and the *in vitro* interaction studies, suggest that the Phu protein is capable of interacting specifically. Furthermore, the *in vitro* interaction studies demonstrate that Phu and CK2α can interact directly.

Nucleotide and predicted amino acid sequences of Phu - The 1469 bp Phu cDNA was determined, and we discovered a large open reading frame (ORF), encompassing nucleotides 1 to 1391, which was in frame with the pACT-GAL-4_{AD}. The ORF was terminated by a TGA stop codon at nucleotides 1392-1394. Near the 5' end, we observed two successive putative ATG

start codons starting at nucleotide 165, of which the second more closely resembles the Kozak consensus sequence (48). The predicted amino acid sequence of the ORF (bp 165 to bp 1391) (Fig. 4.3) encodes a protein of 409 amino acids with a theoretical molecular mass of 46,236 Da. Notably, this predicted size is consistent with that observed for the *in vitro* translation product observed previously (Fig. 4.2).

Screening of the Phu nucleotide or predicted amino acid sequences using the "BLAST" programs (49) (available at: "http://www.ncbi.nlm.nih.gov/") identified only one significant match. The C-terminal domain of Phu (encompassing amino acids 338-409 of the deduced amino acid sequence) displayed 95% identity with the deduced amino acid sequence from a partial cDNA encoding an ORF of 72 amino acids which was previously identified as a putative c-Jun leucine zipper interactive mouse protein identified as "cDNA JZA-20 / pir B46132" (50).

Analysis of the predicted amino acid sequence of Phu by "ProfileScan" (http://www.isrec.isb-sib.ch/cgi-bin/PFSCAN) and "Pfam" (http://www.sanger.ac.uk/cgi-bin/Pfam) identified a Pleckstrin Homology (PH) domain located between amino acid residues 21-132 of the Phu protein (Fig. 4.3).

Northern Blot Analysis - In order to determine whether we had isolated a cDNA encoding the full length Phu protein, we probed a Human Multiple Tissue Northern blot (Clontech)

using a fragment of the Phu cDNA. This probe detected a major transcript of approximately 1.5 kb, as well as an additional transcript of significantly lower intensity at 4.4 kb. The 1.5 kb transcript size is consistent with the size of the Phu cDNA isolated from pACT. The highest amount of the 1.5 kb transcript, was seen in the mRNA from Skeletal Muscle and Heart tissues. Intermediate transcript levels were observed in Brain, Placenta and Lung tissues, while lower transcript levels were seen in the Liver, Kidney and Pancreas tissues. We also probed the same Northern with CK2α, CK2α', and CK2β (Fig. 4.4, panels B, C, D, respectively), in order to compare expression levels of the different mRNAs throughout the various human tissues. Although the expression patterns between Phu, CK2α and CK2α' were similar, there were noticeable differences. The transcript level for CK2α (Fig. 4.4, panel B) was weaker in the lung lane, but stronger in both the kidney and pancreas lanes as compared to the Phu blot (Fig. 4.4, panel A). The transcript level for CK2α' (Fig. 4.4, panel C) was much stronger in the pancreas lane, and stronger in the placenta lane. Although, the significance of the differences in transcript levels is unclear, it is clear that we could easily detect the mRNA of Phu, as well as CK2α and CK2α' in a variety of tissues. CK2β mRNA was readily detected in a fairly uniform fashion in all tissues, except for an apparently lower transcript level detected in Lung (Fig.

4.4, panel D).

To confirm that we indeed possessed a cDNA encoding for the full length Phu protein, we screened the Phu nucleotide sequence against tentative human consensus sequences (THC) through "http://www.tigr.org/". We identified an expressed sequence tag (EST) with a reported size of 1.7 kb that we obtained from ATCC (#410320 / H14297). The nucleotide sequence of this clone (not shown) revealed that a large central region was completely identical to the nucleotide sequence of Phu which encompassed the entire 409 amino acid coding ORF of Phu. The major differences were the existence of additional sequence at both the 5' and 3' ends of the EST H14297 sequence. Importantly, at the 5' end of this EST, an in-frame TGA stop codon was found with no intervening ATG codons between it and the putative start codon(s) of the ORF identified in Phu. At the 3' end of EST H14297, an "AT" rich region was identified, which was not found in the Phu nucleotide sequence. Taken together, these results suggest that the cDNA encoding the full length Phu protein has been isolated.

Detection of a 50 kDa protein with anti-Phu antiserum - In order to study the Phu protein, polyclonal rabbit antibodies directed against a GST fusion protein encoding the C-terminal 102 amino acids of Phu were raised as described in "Experimental Procedures". The anti-Phu

antisera were able to clearly detect a single band of 50 kDa in a Western blot analysis from immunoprecipitates obtained using the same anti-Phu antisera. (Fig. 4.5A, lanes 1 and 2). A 50 kDa band is similarly detected when cell extracts are directly analysed on Western blots with either of the anti-Phu antisera (data not shown). Importantly, the 50 kDa band was not evident when immunoprecipitations were performed with pre-immune sera from either of the rabbits (Fig. 4.5A, lane P). Moreover, the 50 kDa band is effectively competed away upon Western blot analysis using anti-Phu sera in the presence of GST-Phu-CTD (Fig. 4.5C), but not when these blots are performed in the presence of GST (not shown). Overall, these results support the conclusion that the antisera from either of the immunized rabbits specifically recognize the 50 kDa Phu protein.

Measurement of CK2 activity in anti-Phu immunoprecipitates - Immunoprecipitates of Phu from Saos-2 cells were subsequently analysed for kinase activity towards the specific CK2 substrate peptide " RRRDDDSDDD " to determine whether there are interactions between CK2 and Phu in mammalian cells. As illustrated in Figure 4.6, immunoprecipitates performed with anti-Phu antiserum from either of the immunized rabbits displayed CK2 activity that was approximately 10-fold above the background activity that was observed in immunoprecipitates performed with pre-immune

serum. These results provide strong evidence for interactions between CK2 and Phu in mammalian cells. The CK2 activity that was measured in anti-Phu immunoprecipitates represents a relatively low percentage (less than 4%) of the activity that was measured in anti-CK2β immunoprecipitates. It is important to note that this estimate is based on the kinase activities that were measured in immune-complex kinase assays performed using antibodies (anti-Phu or anti-CK2β) that may exhibit differences in immunoprecipitation efficiency. Nevertheless, these results suggest that Phu/CK2 complexes represent a relatively small fraction of the total cellular CK2.

Expression of EGFP-fusion proteins encoding Phu - Based on the evidence from co-immunoprecipitation assays which indicate that Phu and CK2 interact in mammalian cells, we were interested in examining the subcellular distribution of Phu, since we believed that Phu could function as a protein that recruits CK2 to a particular cellular location. To achieve this, we prepared expression constructs encoding the entire ORF of Phu as an Enhanced Green Fluorescent Protein (EGFP)-fusion protein, as well as the final C-terminal 102 amino acids of Phu which lack the Pleckstrin-Homology domain. The resulting EGFP-Phu fusion proteins possessed the EGFP moiety at their respective N-termini. As a prelude to examining the localization of these protein

within cells, we examined their expression by Western blot analysis of transfected Saos-2 cell lysates using anti-Phu antiserum or anti-GFP antiserum (Figure 4.7). As expected, anti-GFP antibodies (Fig. 4.7C) detected EGFP as well as EGFP-Phu-CTD and EGFP-Phu (lanes 1,2,3 respectively). The anti-Phu antibodies (Fig. 4.7B) detect bands in both lanes 2 and 3 that appear to be identical to the bands that are detectable with anti-GFP. There is also a band of approximately 50 kDa detected by anti-Phu antibodies that is most evident in lane 4 (non-transfected Saos-2 cells). This 50 kDa band is not recognized by either the Pre-immune or the EGFP antibodies, and can be competed away with GST-Phu-CTD (data not shown). Based on the similarities of this band with that seen previously, it is likely that this 50 kDa band is the endogenous Phu.

Co-Immunoprecipitation of EGFP-Phu and HA-CK2α - To further characterize the EGFP-Phu fusion proteins, we examined the ability of these proteins to interact with CK2 α in mammalian cells using co-immunoprecipitation assays. Moreover, we were interested in determining whether Phu would exhibit selective interactions with CK2α, and not with CK2α', as we had observed in yeast two hybrid assays. To achieve this objective, we sought to co-transfect cells with a construct encoding EGFP-Phu together with constructs encoding epitope-tagged CK2α or epitope-tagged CK2α'. Prior

to immunoprecipitation of HA-tagged proteins or EGFP fusion proteins, lysates from transfected cells were tested by Western blot analysis to ensure that exogenous EGFP-Phu and HA tagged proteins were being expressed at comparable levels (data not shown). Consistent with our previous studies, we observed that the levels of expression of HA-CK2α were much lower than that observed with the other HA tagged CK2 constructs (14). Anti-HA immunoprecipates were examined on Western blots with anti-Phu antiserum (Fig. 4.8A). Alternatively, immunoprecipitates performed with anti-Phu serum were analysed with anti-HA on Western blots (Fig. 4.8B). In panel A, the EGFP-Phu fusion protein can be visualized in a transfected cell lysate (lane 1), but not in a lysate from cells transfected with empty vector (lane 3). The EGFP-Phu protein is also present in anti-HA immunoprecipitates (Fig. 4.8A) derived from cells co-transfected with EGFP-Phu and either HA-CK2α (lane 4), CK2α-HA (lane 5), or HA-CK2α′/α (lane 7), but not in immunoprecipitates from cells transfected with EGFP-Phu and either HA-CK2α′ (lane 6) or empty vector (lane 3).

In the experiment illustrated in Fig. 4.8B, Saos-2 cells were transfected with EGFP-Phu together with an empty vector (lanes a,b), with HA-CK2α′/α (lanes c,d) or with HA-CK2α′. A band of approximately 45 kDa corresponding to HA-CK2α′/α is present in the cell lysate (lane c) and in the anti-Phu immunoprecipitate (lane d). By comparison, a band

of approximately 40K corresponding to HA-CKα' is detected in a transfected cell lysate (lane e), but not in the corresponding anti-Phu immunoprecipitate. Overall, these results demonstrate co-immunoprecipitation of EGFP-Phu with HA-CK2-α'/α, but not with HA-CK2- α'. These results are consistent with the conclusion that Phu does not interact with CK2α', and that the CTD of CK2α is critical for the interaction between Phu and CK2α. In addition, the results of the co-immunoprecipitation assays suggest that the EGFP-Phu protein retains the capacity to interact with CK2α in mammalian cells.

Subcellular localization of EGFP-Phu constructs by fluorescence microscopy - To examine the subcellular localization of Phu in mammalian cells, we examined Cos 7 cells that had been transfected with constructs encoding EGFP, EGFP-Phu, or EGFP-Phu-$CTD_{308-409}$ by fluorescence microscopy. With EGFP, we observed general cellular fluorescence with very prominent nuclear fluorescence (Fig. 4.9, top row). By comparison, EGFP-Phu does not exhibit prominent nuclear fluorescence, and appears to localize chiefly to discrete areas of the cell consistent with plasma membrane and/or membrane ruffles (Fig. 4.9, middle row). The deletion mutant, EGFP-Phu-$CTD_{308-409}$ displays a general cellular distribution, similar to that observed with EGFP, however, we have observed areas of intense fluorescence near

the nucleus (Fig. 4.9, bottom row). Overall, these results suggest that Phu contains information which is able to affect the cellular distribution of EGFP. It also appears that the N-terminal domain of Phu, which contains the Pleckstrin Homology domain, appears to be required for the plasma membrane staining that is observed.

DISCUSSION

In this study, we have reported the identification of a novel protein of previously unknown function which we have called Phu. Using the yeast two-hybrid system we have observed that Phu is able to interact with the CK2α, but not the CK2α', isoform of protein kinase CK2. Our results also suggest that the unique CTD of CK2α is required, but is not by itself sufficient, for CK2/Phu interactions. In co-immunoprecipitation experiments using co-transfected Saos-2 cells expressing EGFP-Phu epitope tagged CK2α constructs, we were able to co-immunoprecipitate EGFP-Phu with HA-tagged CK2α or CK2α'/α. Importantly, co-immunoprecipitation of Phu and HA-CK2α'/α was consistently observed with either anti-HA antibodies or with anti-Phu antibodies, while co-immunoprecipitation of EGFP-Phu and HA-CK2α' was never observed. These results support the yeast two-hybrid observation that Phu does not interact with CK2α', and

further suggest that the interaction between Phu and CK2α is mediated through the unique carboxy-terminal-domain of CK2α. We were unable to examine the interaction between EGFP-Phu and HA-CK2α in co-immunoprecipitation experiments due to the consistently low expression levels of HA-CK2α in either Saos-2 or Cos 7 cells. Although we have previously observed that HA-CK2α is expressed at dramatically lower levels than HA-CK2α', we do not understand the basis for this observation.

On the basis that our two-hybrid screens were performed to identify proteins that interact with the catalytic subunits of CK2 yielded predominantly the regulatory β subunit of CK2, our screens must be classified as successful. Furthermore, since the Phu cDNA was isolated from the screen using the same criteria that yielded CK2β, it is not surprising that Phu appears to be a physiologically relevant CK2 interacting protein. In utilizing the yeast two-hybrid system to identify proteins that interact with CK2, we anticipated that we could isolate novel substrates or regulators of CK2. Regulators of CK2 could be classified as direct regulators (ie. activators or inhibitors of CK2) or indirect regulators that could have the potential to control the cellular activity of CK2 by controlling its localization in cells or recruiting the enzyme to its substrates. In this regard, we have examined the possibility that Phu is a substrate for CK2 but have

been unable to observe any significant phosphorylation of either GST-Phu or GST-Phu-CTD by purified CK2 *in vitro* under conditions that yield extensive phosphorylation of casein (data not shown). Similarly, neither GST-Phu nor GST-Phu-CTD appear to dramatically alter the activity of purified CK2 *in vitro* (data not shown). These results suggest that Phu is neither a substrate nor a direct regulator of CK2 activity. Consequently, the possibility that Phu may be an indirect regulator of CK2 is likely.

The presence of a putative PH domain suggested that Phu may localize to the plasma membrane, since the PH domains of other proteins have been implicated in localizing these proteins to the plasma membrane (51, 52, 53, 54, 55). This prediction is supported by the observations made with EGFP-Phu, in Cos 7 cells (Fig. 4.9), and in Saos-2 cells (not shown). In fact, the location of EGFP-Phu appears to be remarkably similar to that reported for the N-terminal PH domain of pleckstrin (53). In that study, Ma et al., (1997), demonstrated that the N-terminal PH domain expressed in Cos-1 cells associated with peripheral membrane ruffles and dorsal membrane projections, and that the presence of the N-terminal PH domain of pleckstrin is critical for targeting pleckstrin to the plasma membrane (53).

It is intriguing to speculate that Phu may act to target or sequester CK2 to the plasma membrane. This idea would be consistent with a recent report demonstrating that CK2 is

associated with the plasma membrane (21, 33), along with reports of plasma membrane localized CK2 substrates (including spectrin (56), insulin receptor (57), caveolin (58), IGF-II (59), dynamin (60), IRS-1 (61), and most recently CD5 (33). These findings could implicate both Phu and CK2α in membrane associated cellular processes such as receptor-mediated signaling,vesicle transport, and/or cytoskeleton organization. In this regard, CK2 has been previously implicated in insulin signaling (62,63,64) although the nature of its involvement is not clear and somewhat controversial (7,65,66). IRS-1 is a key intracellular regulatory protein that transduces the insulin receptor signal to a variety of proteins (67). It is interesting to note that CK2, the insulin receptor, IRS-1 and dynamin have all been co-localized to caveolae (58,68,69,70,71), and that, like Phu, IRS-1 and dynamin both possess PH domains.

Alternatively, Phu may act to recruit some CK2α in the cytoplasm, which would allow CK2 to phosphorylate some cytoplasmic CK2 substrates, such as: p65(synaptotagmin) (72), furin (73,74), and dynein (75), and regulate intracellular trafficking. Moreover, the observations that CK2α is implicated in the polarized growth of both fission and budding yeast cells (76,77), as well as neuritogenesis in mouse neuroblastoma (N2A) cells (78), suggests a role for CK2 in the organization of the actin cytoskeleton. In fact,

many proteins which interact with the cytoskeletal regulating GTPases (Rac, Rho, and cdc42), possess PH domains, leucine zippers, proline rich regions, and/or kinase domains (79). These protein domains are distributed between Phu (PH domain and Leu zipper) and CK2α (kinase domain and Proline rich region at its CTD). Interestingly, overexpression of EGFP-Phu also appears to change the cell morphology of Cos 7 cells by giving the cells a rounder appearance than what is typically observed in non-transfected or EGFP transfected cells. Suggesting that CK2α/Phu may be involved in cytoskeletal re-arrangement, however a more detailed microscopic analysis is required to further characterize the nature of these morphological changes. Whether CK2α/Phu plays a role in cytoskeletal signaling or re-arrangement remains to be determined, but is worthy of further investigation.

In conclusion, we have identified a protein, Phu, which is able to interact with CK2α. Phu may act to sequester a fraction of the total cellular CK2 in the cytoplasm, thus allowing CK2 to phosphorylate cytoplasmic/plasma membrane substrates. In this regard, Phu may regulate CK2 activity in a cell in a similar fashion to that of the many modular protein complexes which regulate kinase activity by sequestering these kinases to discrete areas of the cell. (reviewed in 18,55,80,81,82, and 83). Furthermore, the selective interactions between Phu and CK2α provide one more

indication that there may be functional differences between the isozymic forms of CK2.

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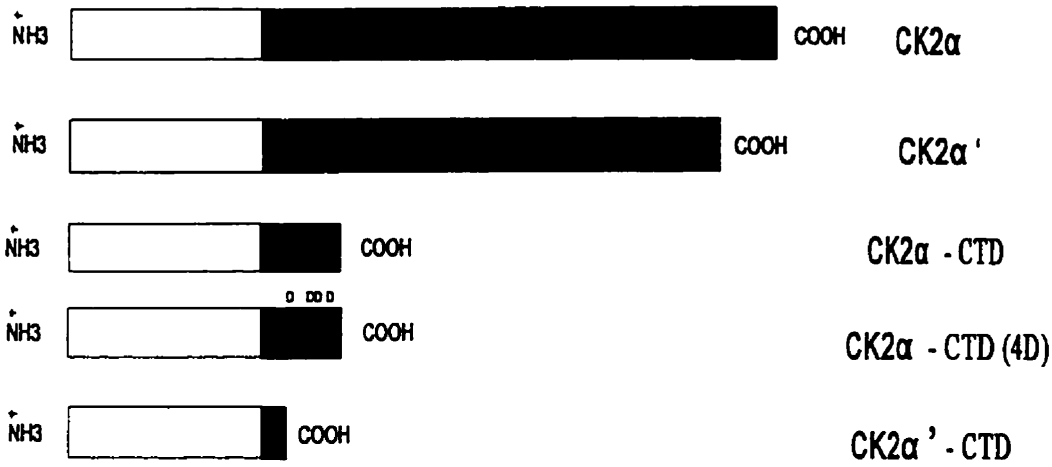
Figure 4.1 **Isolation of CK2 interacting proteins using the yeast two-hybrid system.** A. To isolate cDNAs encoding CK2 interacting proteins, cDNAs encoding full length CK2α or CK2α' were expressed in yeast as fusions with the DNA binding domain of GAL4 using the plasmid pGBT9 as described in Experimental Procedures. Constructs encoding GAL4 DNA binding domain fusions encoding the unique C-terminal domains of CK2α and CK2α', designated CK2αCTD and CK2α'CTD, were also prepared. The CK2αCTD(4D) fusion is a mutant where each of the mitotic phosphorylation sites on CK2α have been mutated to aspartic acid residues. B. Yeast expressing each of the constructs shown in A were transformed with an EBV-transformed human lymphocyte cDNA library constructed in the vector pACT to express fusions with the transcriptional activation domain of GAL4. Transformation efficiency was determined by growing transformants on Trp and Leu deficient media. Colonies that exhibited growth on media deficient in Trp, Leu, His, and Ade were considered to be positives. Positives were subsequently examined for the expression of CK2β. Those that failed to grow were not tested. C. The pACT plasmid from one positive that did not contain CK2β (pACT-38) was transformed into yeast to test for interactions with a variety of independent GAL4-DNA binding domain fusions that were expressed using pGBT9 or pAS1. Positive interactions were indicated by the ability of transformants to grown on media deficient in Trp, Leu, His and Ade.

A



B

Table 4.1 - Results of yeast two-hybrid screen

BAIT (Gal4-DNA-BD)	TRANSFORMANTS # cfus on T^-L^- growth media	POSITIVES # cfus on $T^-L^-H^-A^-$ growth media	PREY (Gal4-DNA AD)		
			Not Tested	+ CK2β	- CK2β
CK2α	2.79×10^6	243	7	235	1
CK2α'	1.19×10^6	196	15	181	0
CK2α - CTD	2.51×10^6	0	-	-	-
CK2α-CTD(4D)	3.78×10^6	0	-	-	-
CK2α' - CTD	2.32×10^6	0	-	-	-

C

Table 4.2 - Specificity of interaction between Phu and CK2α

Constructs tested against pACT-Phu	Growth on selective media ($His^-Ade^-Leu^-Trp^-$)
pGBT9	No
pGBT9-CK2α	Yes
pGBT9-CK2α '	No
pGBT9-CK2α-CTD	No
pGBT9-CK2α-CTD (4D)	No
pGBT9-Huntingtin *	No
pAS1-Fragmentin	No
pAS1-Rad7	No

* as described by Kalchman et al., (1996) *J. Biol. Chem.* **271**:19385-19394

Figure 4.2 ***In vitro* interactions between CK2α and Phu.** *In vitro* transcription/translation reactions were performed using a reticulocyte lysate based system with (designated P) or without (designated C) a PCR product that was amplified from pACT-38 as described in Experimental Procedures. Panel A. ^{35}S-labelled reaction products from the *in vitro* transcription/translation reactions were subjected to electrophoresis on 12% SDS-polyacrylamide gels and visualized using a phosphorimager. The major translation product of approximately 50,000 Da is indicated. The positions of molecular weight markers are also indicated and are as follows from top to bottom: 175,000; 83,000; 62,000; 47,500; 32,500; 25,000 and 16,500. *In vitro* translation products were incubated with GST-CK2α or GST coupled to Affigel beads as described in Experimental Procedures. Panel B. Radiolabelled proteins that were retained on either of the respective Affigel beads were subjected to SDS-polyacrylamide gel electrophoresis and visualized as in A. Panel C. Approximately 25% of each supernatant were similarly analysed.

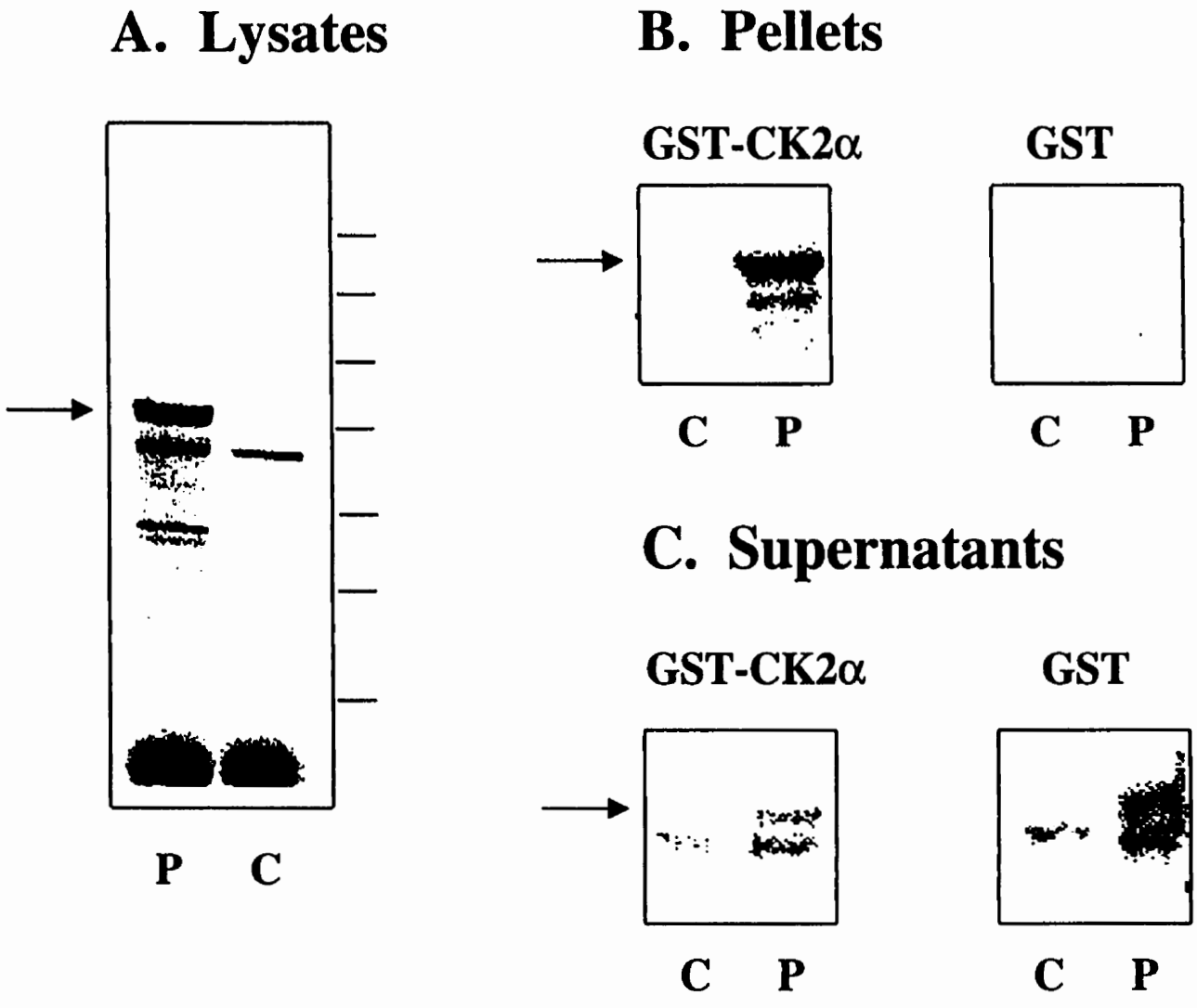

A. Lysates
B. Pellets
GST-CK2α
GST
C P
C P
C. Supernatants
GST-CK2α
GST
C P
C P
P C

Figure 4.3 **Predicted amino acid sequence and schematic representation of Phu.** A. Amino acid sequence of Phu. The predicted amino acid sequence of Phu was determined by sequencing the insert of pACT-38 and was confirmed by sequencing an EST. The pleckstrin homology (PH) domain of phu is highlighted by grey shading. B. Schematic representation of Phu illustrates the presence of an N-terminal pleckstrin homology domain and also shows the region of similarity to that of the predicted amino acid sequence obtained from a partial cDNA previously identified as a c-Jun leucine zipper interacting protein (ref. 50). Residues that form a putative leucine zipper are boxed in.

A

1	M	M	K	K	N	N	S	A	K	R	G	P	Q	D	G	N	Q	Q	P	A
21																		
41																		
61																		
81																		
101																		
121													R	A	K	N	R	I	L	D
141	E	V	T	V	E	E	D	S	Y	L	A	H	P	T	R	D	R	A	K	I
161	Q	H	S	R	R	P	P	T	R	G	H	L	M	A	V	A	S	T	S	T
181	S	D	G	M	L	T	L	D	L	I	Q	E	E	D	P	S	P	E	E	P
201	T	S	C	A	E	S	F	R	V	D	L	D	K	S	V	A	Q	L	A	G
221	S	R	R	R	A	D	S	D	R	I	Q	P	S	A	D	R	A	S	S	L
241	S	R	P	W	E	K	T	D	K	G	A	T	Y	T	P	Q	A	P	K	K
261	L	T	P	T	E	K	G	R	C	A	S	L	E	E	I	L	S	Q	R	D
281	A	A	S	A	R	T	L	Q	L	R	A	E	E	P	P	T	P	A	L	P
301	N	P	G	Q	L	S	R	I	Q	D	L	V	A	R	K	L	E	E	T	Q
321	E	L	L	A	E	V	Q	G	L	G	D	G	K	R	K	A	K	D	P	P
341	R	S	P	P	D	S	E	S	E	Q	L	L	L	E	T	E	R	L	L	G
361	E	A	S	S	N	W	S	Q	A	K	R	V	L	Q	E	V	R	E	L	R
381	D	L	Y	R	Q	M	D	L	Q	T	P	D	S	H	L	R	Q	T	T	P
401	H	S	Q	Y	R	K	S	L	M	*	409									

B

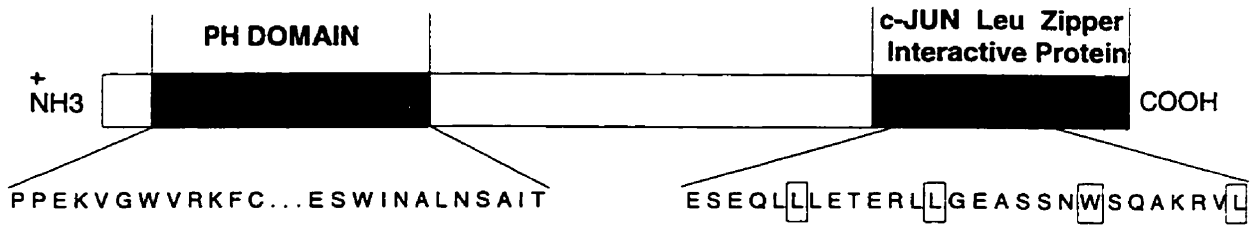


Figure 4.4 **Northern blot analysis.** Expression of Phu (A), CK2α (B), CK2α′ (C) and CK2β (D) in a variety of adult human tissues. Tissues are as follows: Pa (pancreas), K (kidney), M (skeletal muscle), Li (liver), Lu (lung), Pl (placenta), B (brain) and H (heart).

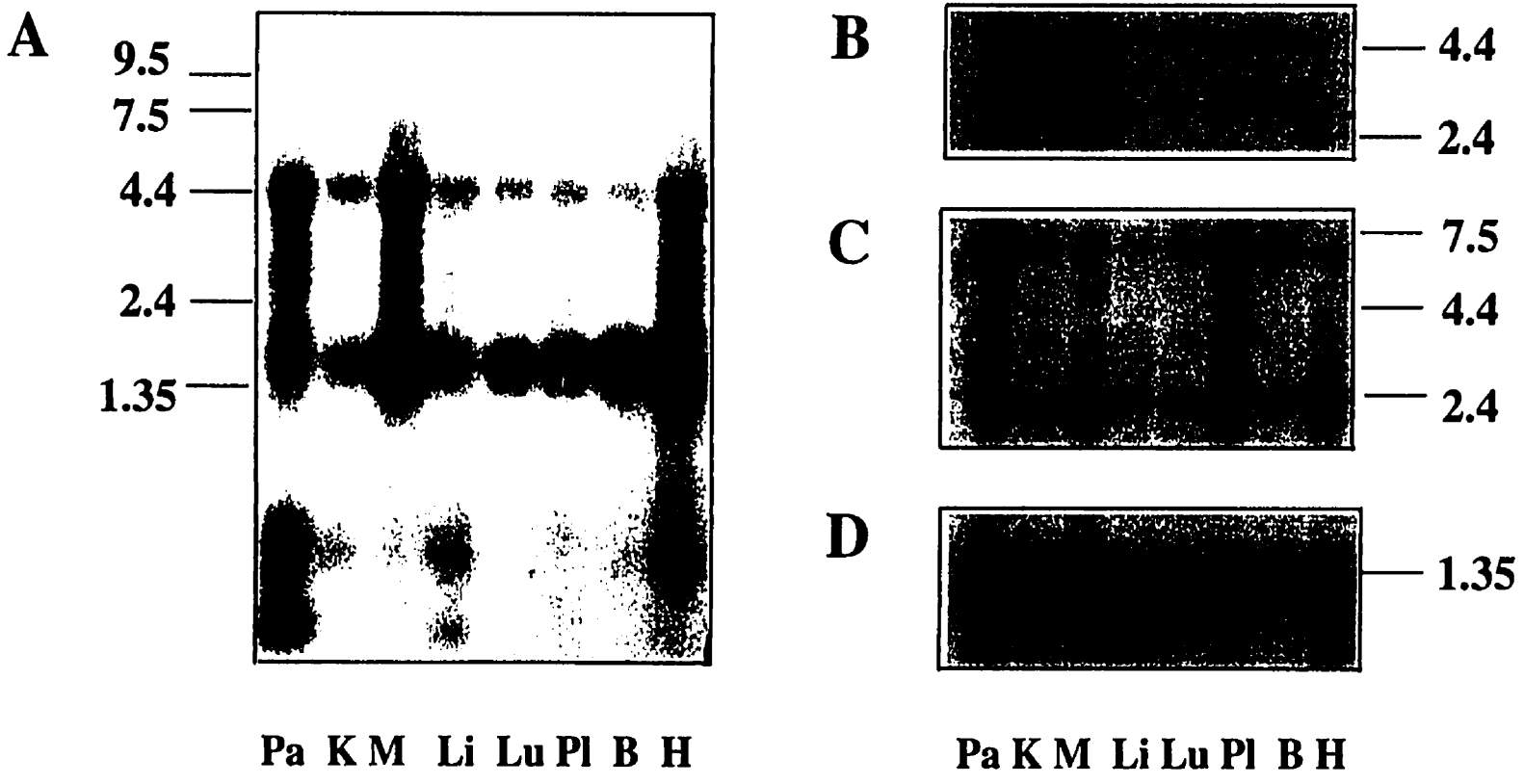
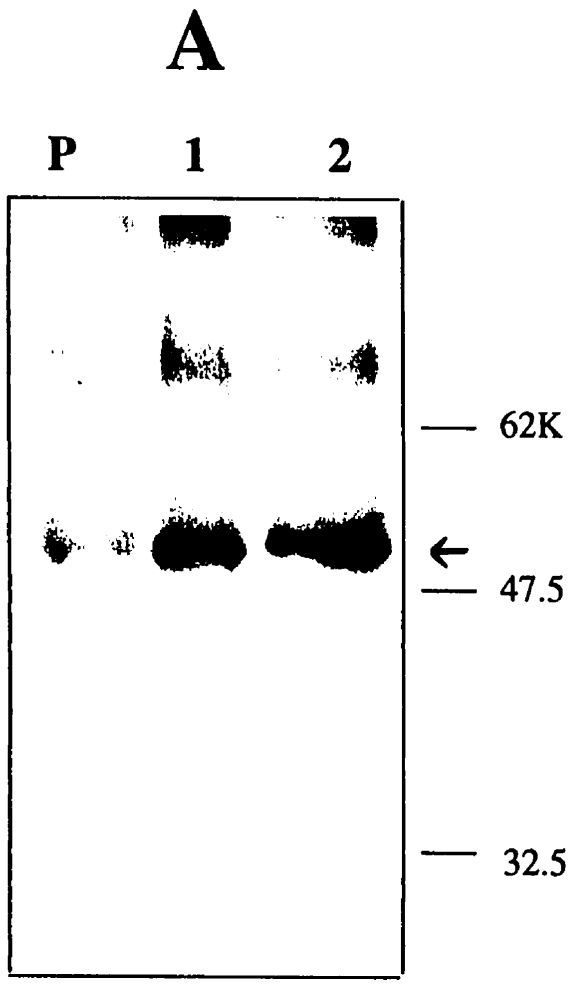

A
9.5
7.5
4.4
2.4
1.35
Pa K M Li Lu Pl B H
B
4.4
2.4
C
7.5
4.4
2.4
D
1.35
Pa K M Li Lu Pl B H

Figure 4.5 **Immunoblot analysis of anti-Phu immunoprecipitates.** Panel A. Extracts were prepared from human osteosarcoma Saos-2 cells using RIPA buffer as described in Experimental Procedures and were subjected to immunoprecipitation with antiserum derived from either of 2 rabbits (designated 1,2) that were immunized with GST-Phu-CTD or with pre-immune serum (designated P). Immunoprecipitates were subjected to electrophoresis on 12% SDS-polyacrylamide gels and analysed by immunoblotting using anti-Phu antiserum. A band of approximately 50 kDa (indicated by an arrow) that is present in the immunoprecipitates that were performed with either of the antisera. Molecular weight markers are also indicated and from top to bottom are as follows: 62,000, 47,500, and 32,500. Panels B and C. Anti-Phu immunoprecipitates from Saos-2 cells were subjected to immunoblot analysis using antiserum from either of 2 rabbits that were immunized with GST-Phu-CTD (designated 1,2) or with pre-immune serum obtained from the same rabbits (designated 1P, 2P) in the presence (C) or absence (B) of the GST-Phu-CTD fusion protein as a competitor.



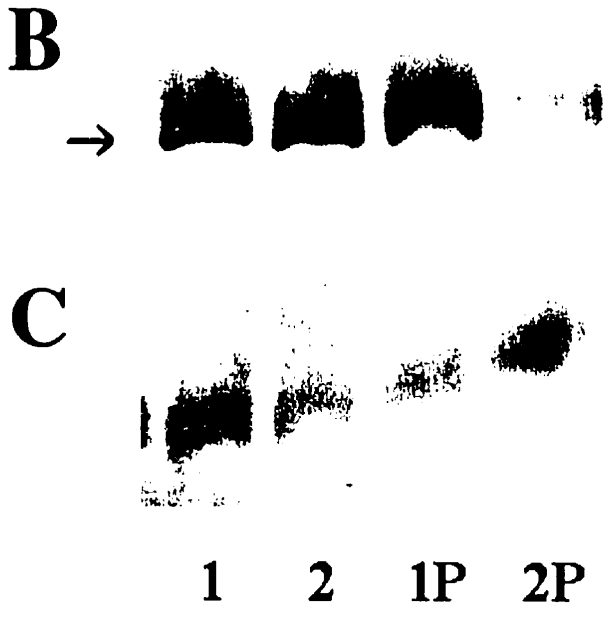


Figure 4.6 **Measurement of CK2 activity in anti-Phu immunoprecipitates.** Extracts were prepared from human osteosarcoma Saos-2 cells using NP40 lysis buffer and were subjected to immunoprecipitation with antiserum from either of 2 rabbits that were immunized with GST-Phu-CTD (designated as 1,2) or with pre-immune serum obtained from either of the same rabbits (designated as 1(pre), 2(pre)). Immunoprecipitates were utilized in kinase assays using the specific CK2 substrate peptide RRRDDDSDDD to measure CK2 activity. Incorporation of ^{32}P phosphate into synthetic peptide from ^{32}P-ATP was determined by P81 filter paper assay as described in Experimental Procedures. Results represent the average (+/- range) of duplicate determinations and are representative of 3 independent experiments.

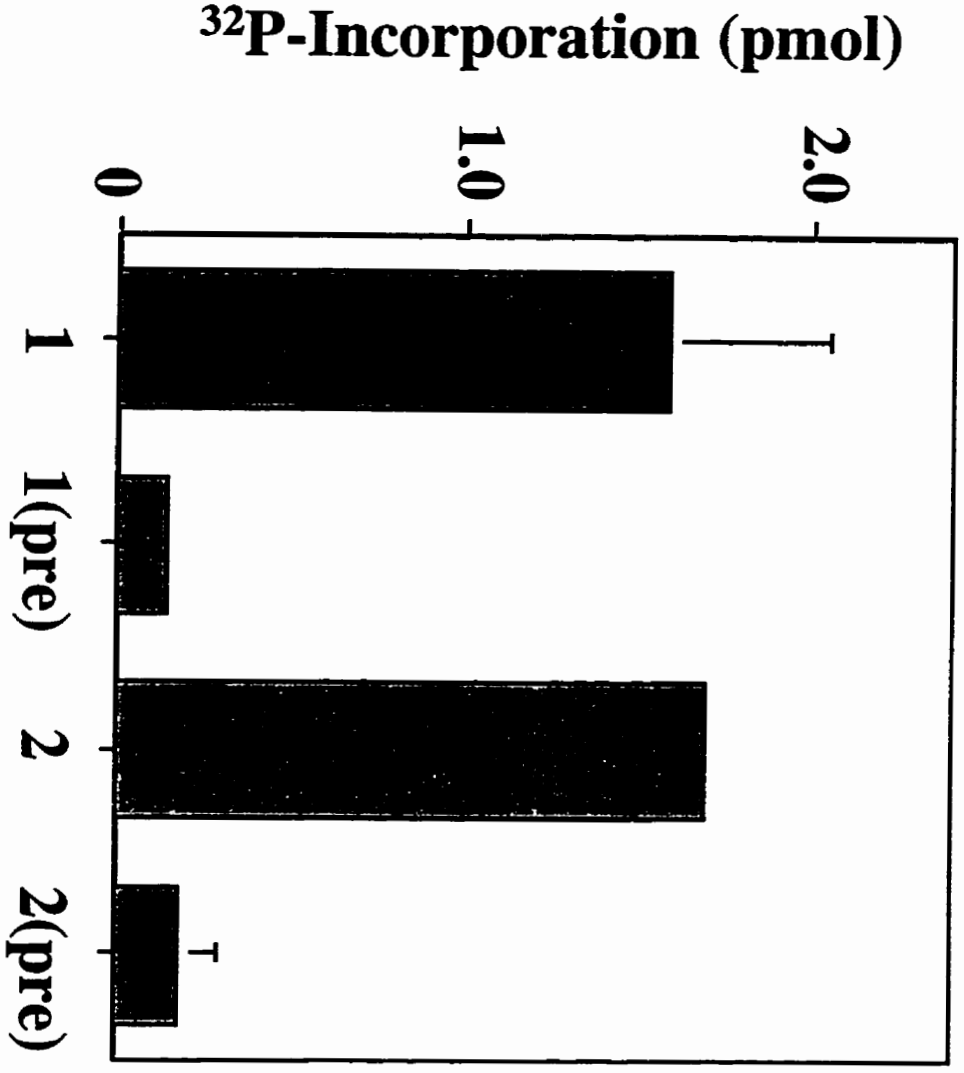

^{32}P-Incorporation (pmol)
0
1.0
2.0
1
1(pre)
2
2(pre)

Figure 4.7 **Immunoblot analysis of EGFP-Phu fusion proteins.** Human osteosarcoma Saos-2 cells were transfected with pEGFP-C2 (lane 1), pEGFP-C2-Phu-CTD (lane 2), pEGFP-C2-Phu (lane 3) or were not transfected (lane 4). Lysates were subjected to electrophoresis and analysed on immunoblots with pre-immune serum (panel A), with anti-Phu (panel B) or with anti-GFP (panel C). The EGFP band is indicated by an arrow, the EGFP-Phu band is indicated by an arrow-head, while the Phu band is marked with an asterisk (*). Molecular weight markers from top to bottom are as follows: 97,400; 66,000; 46,000; 30,000.

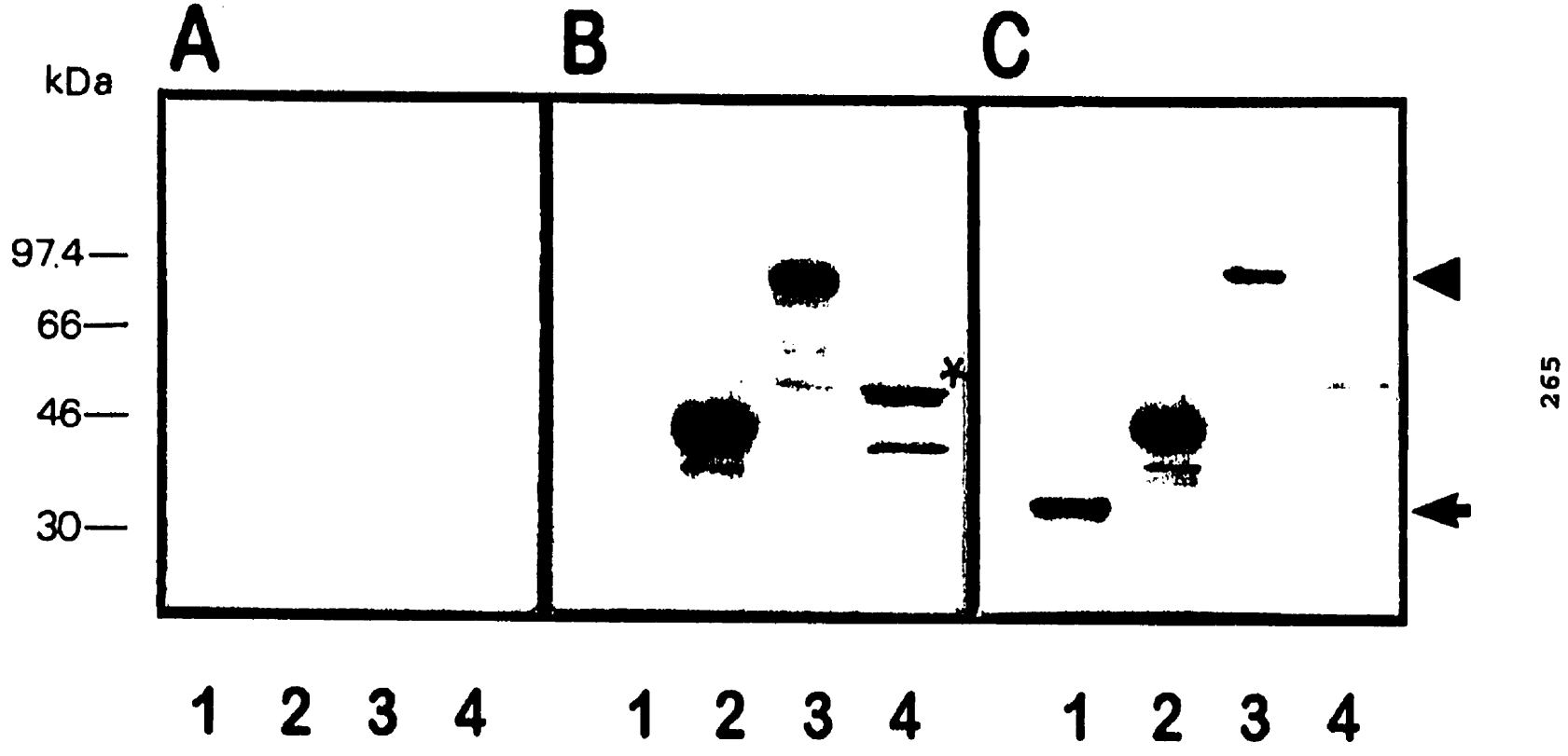

A
B
C
kDa
97.4
66
46
30
*
1 2 3 4 1 2 3 4 1 2 3 4
265

Figure 4.8 **Co-immunoprecipitation of EGFP-Phu and CK2.** Human osteosarcoma Saos-2 cells were transfected with pEGFP-C2-Phu and the indicated CK2 constructs.

A. Immunoprecipitations were performed with anti-HA (ie. 12CA5) antibodies and immunoprecipitates were analysed on immunoblots with anti-Phu. Lane 2 (blank), lane 3 (pRC-CMV), lane 4 (HA-CKα), lane 5 (CK2α-HA), lane 6 (HA-CK2α'), and lane 7 (HA-CK2α'/α-CTD). For comparison purposes, a lysate from cells transfected with pEGFP-C2-Phu is also shown (Lane 1). The position of EGFP-Phu is indicated. B. Immunoprecipitations were performed with anti-Phu antibodies and analysed on immunoblots with anti-HA. Lysates (L) (lanes a, c, and e) and immunoprecipitates (IP) (lanes b, d, and f) are shown. Lanes a & b (pRC-CMV), lanes c & d (HA-Ckα'/α-CTD), and lanes e & f (HA-CKα'). The position of HA-CK2α'/α-CTD is indicated.

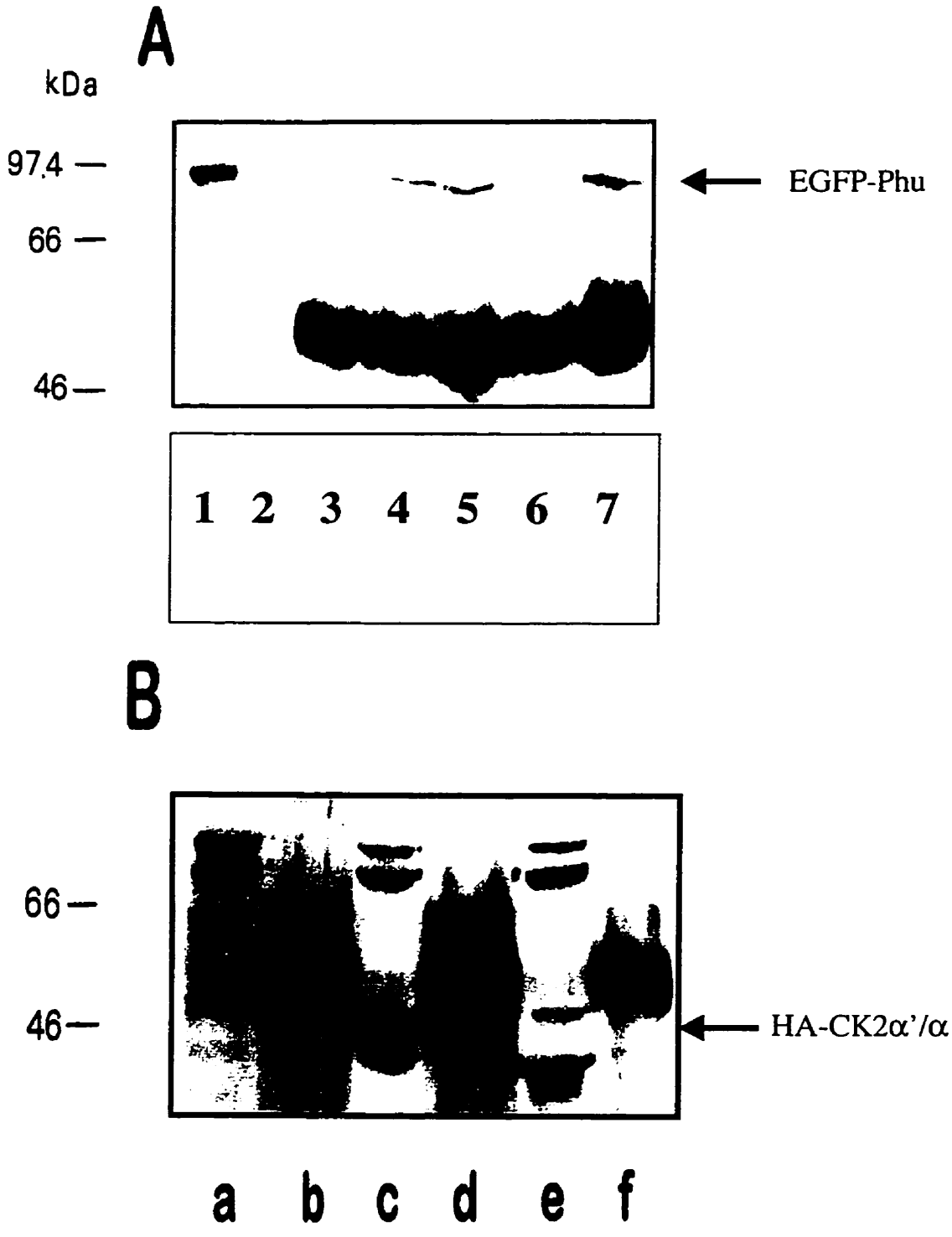
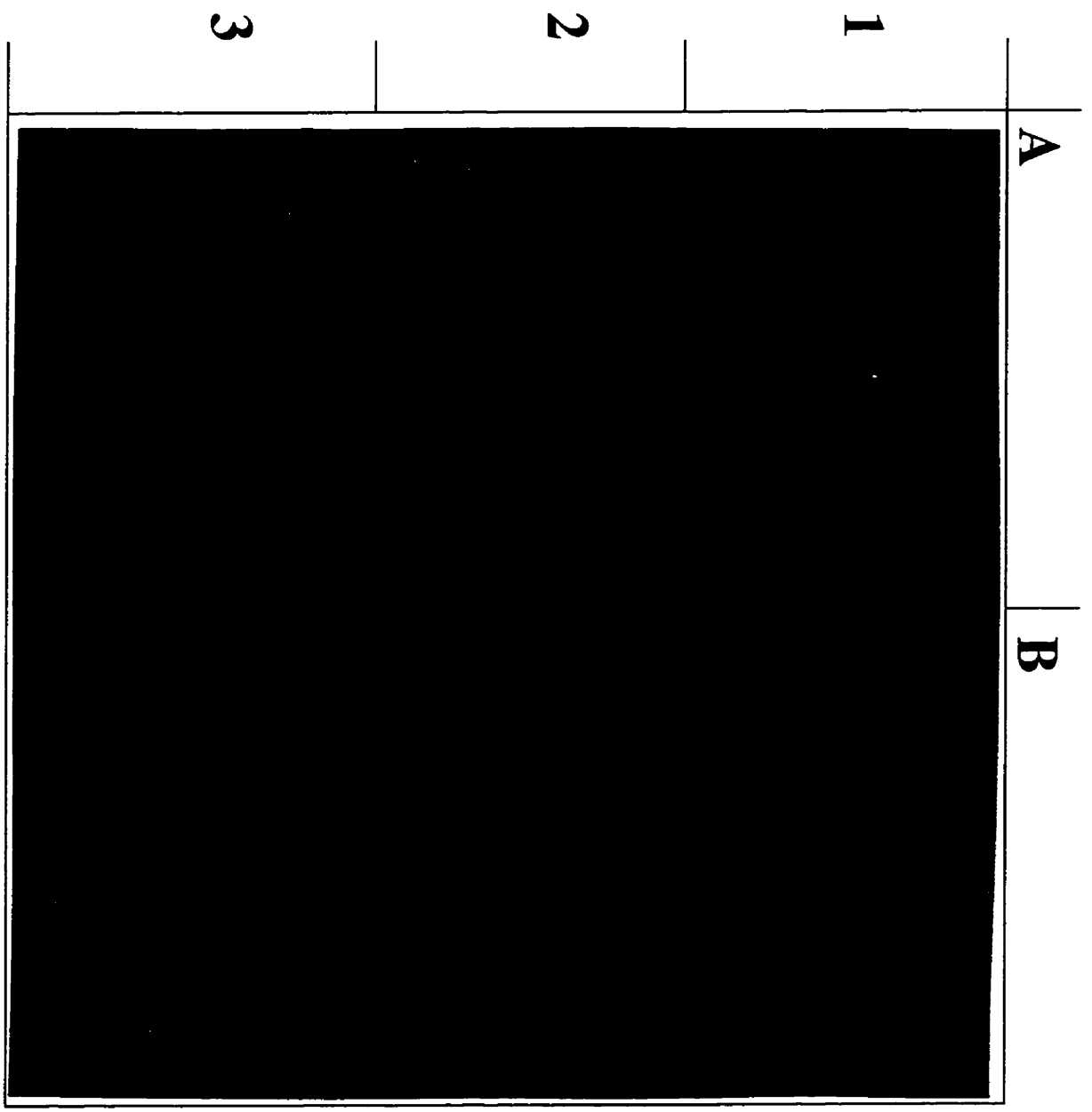

A
kDa
97.4
66
46
EGFP-Phu
1 2 3 4 5 6 7
B
66
46
HA-CK2α'/α
a b c d e f

Figure 4.9 **Subcellular localization of EGFP-Phu fusion proteins.** Human osteosarcoma Saos-2 cells were transfected with pEGFP-C2 (Row 1 - top panels), pEGFP-C2-Phu (Row 2 - middle panels) or pEGFP-C2-Phu-CTD (Row 3 - bottom panels) and examined by fluorescence microscopy as described in Experimental Procedures to detect the EGFP fusion proteins (Column A - left panels). Cells were also stained with DAPI to visualize the nuclei (Column B - right panels).


3
2
1
A
B

CHAPTER 5

DISCUSSION

As stated earlier, our laboratory hypothesized that CK2α and CK2α' exhibit functional specialization or differences, and that these differences may be reflected in cell cycle dependent expression, post-translational modifications or protein interactions. In this section I will summarize the observations and findings reported in this thesis, and place them in context with the findings and observations of other groups, while outlining relevant future studies.

CK2 AND CELL CYCLE PROGRESSION -

Using genetic and biochemical approaches, various labs have clearly shown that CK2 is an important participant in cell proliferation and cell cycle progression. Work done by many researchers has led to the identification of a category of genes which encode for proteins involved in cell cycle checkpoints, which ensure the proper order of cell cycle events. Cell cycle checkpoints can negatively or positively control cell cycle progression (1), therefore, the importance of characterizing key components of cell cycle checkpoints will not only lead to a better understanding of

cellular biology events, but may also lead to the development of more efficacious treatments against human proliferative diseases such as cancer. In fact, a recent report demonstrated that response by human colon cancer cells to anticancer therapeutics is dependent on the integrity of their cell-cycle checkpoints (2). This group reported that a significant fraction of p21 checkpoint-deficient tumor cells could be cured with gamma-radiation treatment, as compared to tumor cells having an intact p21 cell cycle checkpoint (2). The implications of these observations towards treatment of cancer cells are that a combinatorial treatment using a compound able to disrupt key cell cycle checkpoint regulating proteins, may improve the efficacy of subsequent radiation therapy. An example of compounds targeting key cell cycle targets are the CDK (cyclin dependent kinase) inhibitors (CDIs). CDIs are currently being evaluated as potential therapeutic agents in cancer treatment (3). Notably, these CDK inhibitory compounds have been demonstrated to inhibit cell growth of many tumour cell lines, by causing cells to arrest in both G_1 and late G_2/early prophase stages of the cell cycle (3). The appeal of CDIs as antitumour agents is that they possess certain desirable properties such as: i) high specificity, which is more than likely to result in fewer secondary effects and ii) the ability to induce apoptosis in actively dividing cells (3). In a similar vein, if we consider CK2 as

an important participant in cell cycle progression, it may prove to be good target for regulation by effector compounds. Obviously, much more research regarding CK2 and its role on cell cycle progression needs to be accomplished before such a concept is implemented in the clinic.

Summary-

In chapter 2 of this thesis we described how we examined the expression levels of the individual subunits of CK2, along with CK2 activity, during cell cycle progression. This was accomplished using various synchronization methods such as, serum-induction of serum-deprived primary cells, centrifugal elutriation, or mitotic arrest using Nocodazole. These methods allowed us to obtain population of cells throughout the G_0 -> M stages of the cell cycle. This work was primarily motivated by the need for a systematic examination of CK2 during cell cycle progression, since previous studies by various laboratories had not examined the protein levels of the CK2α' subunit, or had obtained conflicting results with respect to CK2 activity.

When we compared CK2 activity and expression levels from transformed cells versus primary cells, our results were consistent with reports from numerous laboratories, in that we observed elevated levels of CK2 activity and expression in the transformed cells compared to the primary cells (4). Upon examination of CK2 activity during cell cycle

progression, our results were contrary to reports by Carroll and Marshak, (1989), (5), and Marshak and Russo, (1994) (6), but in agreement with Schmidt-Spaniol et al., (1993) (7) and most recently with Orlandini et al., (1998) (8), in that we did not notice any major fluctuations or oscillations in CK2 activity during cell cycle progression. With respect to CK2 expression, we did not observe any major relative differences in expression between CK2α and CK2α' at different stages of the cell cycle. However, we did observe a marked reduction in the protein levels, of both CK2α and CK2α', in serum-deprived quiescent cells, compared to cycling cells. Interestingly, the levels of both CK2α and CK2α' increased co-ordinately upon serum stimulation of quiescent cells. In similar studies, Schmidt-Spaniol et al., (1993), who did not examine the protein levels of CK2α', detected no changes in protein levels of CK2α in primary human fibroblasts or human fibrosarcoma cell extracts, but did notice increased levels of CK2β in serum-stimulated quiescent primary human fibroblasts (7). Of note is that, although we were able to readily detect CK2β in the transformed cell lines, we were unable to consistently detect CK2β protein levels in the primary cell line. The differences in results between our lab and that of Schmidt-

Spaniol, et al., (1993) could reflect discrepancies arising from the use of different cell lines, passage number, preparation of sample cell extracts, antibodies, and/or synchronization methods. Recently, Orlandini et al., (1998) reported modest increases of CK2α' and CK2β, and very modest increase of CK2α mRNA levels associated with serum-induction of quiescent NIH-3T3 cells (8). The CK2α and CK2α' data is consistent with our findings, while the CK2β data agrees with some of the results from Schmidt-Spaniol, et al., (1993) (7).

Altogether, our results, along with those of other laboratories, indicate that there is an associated increase in the levels of the individual CK2 subunits which is concomitant with cell cycle entry. This suggests that upregulation of the expression of CK2 subunits may be important for cell entry.

Intriguingly, we observed an increase in the levels of CK2β protein in chicken B-cells arrested in mitosis with Nocodazole. We are uncertain of the significance of this phenomenon, and do not know whether this reflects an increase in CK2β synthesis or a stabilization of pre-existing CK2β. There are indications that the latter may be correct, since Lüscher and Litchfield, (1994) reported that CK2β which is not incorporated into CK2 holoenzyme is

quickly degraded by a non-lysosomal process (9). Moreover, Allende and Allende, (1995) observed similarities between a region of CK2β and the cyclin destruction box of cyclins A and B (10). The presence of a cyclin destruction box, which is crucial for degradation of the mitotic cyclins, may indicate that CK2β shares similar degradation mechanisms to the mitotic cyclins. The degradation of cyclin B, and subsequent inactivation of $p34^{cdc2}$, are important steps for the completion of mitosis. This process is dependent on the successful activation and targeting of $p34^{cdc2}$/cyclin B to the mitotic spindle, concomitant with ubiquitination and eventual degradation of cyclin B (11,12,13,14). The observation that CK2β can be ubiquitinated in cells (personal communication D. Litchfield), and that CK2β, as well as CK2α and CK2α', are associated with the mitotic spindle (15,16), re-inforce the idea that CK2β levels could be regulated through similar degradation mechanisms which affect the mitotic cyclins. Furthermore, the importance of regulating cellular levels of CK2β for cell cycle exit was shown in experiments by Roussou and Draetta, (17), who demonstrated that overexpression of CK2β in *Schizosaccharomyces pombe* inhibited cell growth and cytokinesis. Altogether, these observations suggest that the

regulation of cellular levels of CK2β may be important for completion of mitosis and cell cycle exit.

Another intriguing observation was that there was an activation of CK2, in the presence of immunopurified $p34^{cdc2}$, that was independent of phosphorylation. A similar phenomenon has been reported by Meggio et al., (1995)(18), and suggests an intimate relationship between CK2 and $p34^{cdc2}$. A relationship between CK2 and $p34^{cdc2}$ in cells was previously demonstrated by Litchfield et al., (1992), who showed that CK2α and CK2β, but not CK2α', are phosphorylated in mitotic cells, by $p34^{cdc2}$ (19). The observation that CK2α, but not CK2α', is phosphorylated in mitotic cells implies that CK2α and CK2α' are independently regulated in cells. This further suggests that functional differences exist between CK2α and CK2α', particularly during mitosis. Since the mitotic phosphorylation of CK2 does not appear to modulate CK2 activity (described in chapter 2), and as an initial step towards eventually understanding the role of the mitotic phosphorylation of CK2, we identified the mitotic sites of phosphorylation of CK2α. The approach we took involved the use of synthetic peptides and GST-CK2α-CTD fusion proteins in which the suspected phosphorylation sites were mutated to non-phosphorylatable alanine residues as described in Chapter 2.

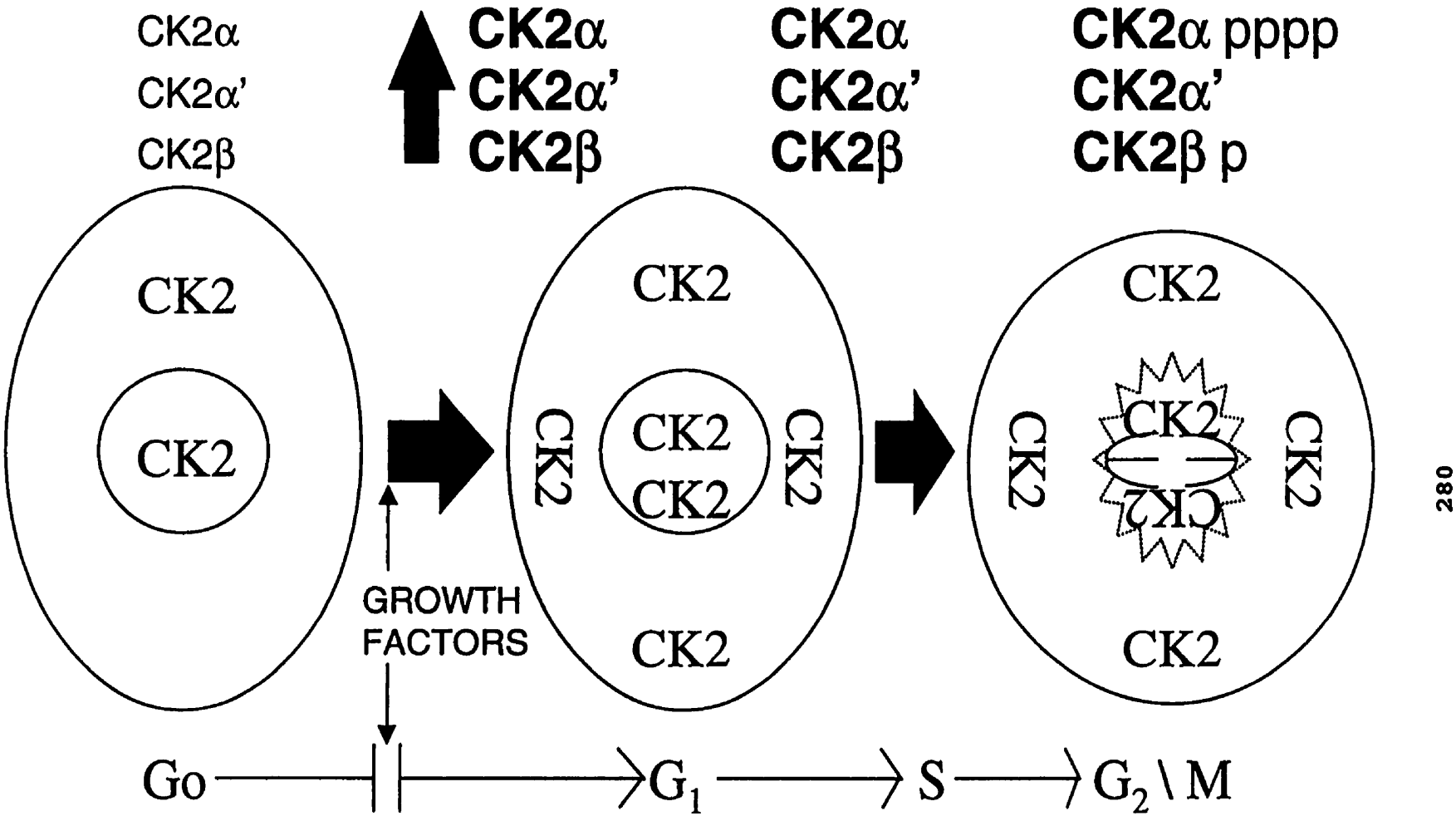
Using this approach, we were able to successfully identify Thr^{344}, Thr^{360}, Ser^{362}, and Ser^{370} as the sites of mitotic phosphorylation in cells, and that $p34^{cdc2}$ is the kinase responsible for the phosphorylation. The validity of the identification that these residues are the mitotic sites of phosphorylation is reflected in the ability to express wild type or "non-phosphorylatable mutant" forms of epitope tagged CK2α in mammalian cells, and observe that the wild type, but not mutant forms, is phosphorylated in mitotic cells (personal communication with D. Litchfield).

Implications and Future Directions-

The results obtained in Chapters 2 and 3 reflect a possible role for CK2 in the entry and exit of cell cycle. Entry in the cell cycle could be dictated, at least in part, by increases in the protein levels of CK2α and CK2α'. At the other end of the cell cycle, exit from the cell cycle may be partially regulated by phosphorylation of CK2 by $p34^{cdc2}$. At the present time, the physiological function of this phosphorylation event is unknown, but there is evidence which suggests that phosphorylation of CK2α may promote an interaction with Pin1 (personal communication with D. Litchfield). The identification of the mitotic sites of phosphorylation for CK2α (Thr^{344}, Thr^{360}, Ser^{362}, and Ser^{370}) and

CK2β (Ser^{209}) (19, Chapter 3 of this thesis) is currently allowing researchers to express phosphorylation site mutants in cells in an effort to determine the effects of the mitotic phosphorylation of CK2. Although, as previously stated, these effects are unknown, it is tempting to speculate that the phosphorylation may modulate the affinity of CK2 towards key cellular substrates or affect its cellular localization. Ultimately, phosphorylation of CK2 could lead to an attenuation or complete inhibition of transcriptional, translational, and intracellular trafficking processes in mitotic cells. This is an attractive notion since, as described in the introduction, each of these processes involve proteins which are substrates of CK2. Moreover, since CK2 has been shown to associate with the mitotic spindle (15,16), CK2 may be involved with other mitotic phenomena such as nuclear disassembly, or tubulin re-arrangement.

Fig. 5.1 **CK2 subunits and cell cycle progression.** Diagram of a cell as it passes through the cell cycle. During quiescence (G_0), the protein levels of CK2 subunits are relatively low, and are distributed within the cytoplasm and nucleus. Upon mitogenic stimulation by growth factors, the protein levels of the CK2 subunits increase concomitantly with entry of the cell into the cell cycle. The relative levels of the CK2 subunits remain constant as the cell progresses through the cell cycle. When the cell reaches mitosis (M), a portion of the CK2 subunits associate with the mitotic spindle, and also, the CK2α and CK2β subunits are phosphorylated at their respective C-termini.


CK2α
CK2α'
CK2β
CK2α
CK2α'
CK2β
CK2α
CK2α'
CK2β
CK2α pppp
CK2α'
CK2β p
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CK2 INTERACTING PROTEINS -

The observations that the CTDs of CK2α and CK2α' are highly conserved in higher eukaryotes, do not influence catalytic activity, and do not appear to influence the formation of the CK2 holoenzyme, coupled with the observation that CK2α contains a "P-X-X-P" motif, which is a motif that has been shown to direct protein:protein interaction in other proteins (20,21,22), led us to hypothesize that the CTDs of CK2α and CK2α' may contribute to the regulation of CK2 through protein:protein interactions.

The idea that CK2 activity can be regulated through specific protein associations has been demonstrated with such proteins as CD5 and $p21^{waf1/cip1}$ (23,24). Moreover, with the emerging identification of scaffold, anchoring and docking proteins that can interact and regulate the time and cellular location at which certain kinases are activated (25,26,27), is an appealing concept for the regulation of a kinase, such as CK2, which is involved in a myriad of cellular processes throughout the cell, and whose cellular regulation is poorly understood. Furthermore, the observation that two of the mitotic sites of phosphorylation (T^{360} and S^{362}) within the carboxyl-terminal domain (CTD) of CK2α are situated adjacent to the "P-X-X-P" motif,

suggested that if a protein:protein interaction was mediated through the CTD of CK2α, this interaction could be regulated by the phosphorylation state of CK2α.

Summary-

In chapter 4, I described how our laboratory used the yeast-two hybrid system to identify a novel 50 kDa protein which appears to interact specifically with CK2α, but not with CK2α'. We demonstrated that this novel protein, Phu, interacted directly with CK2α *in vitro*, and co-immunoprecipitated with CK2α in mammalian cells. We further demonstrated that the presence of the CTD of CK2α is an important determinant for the interaction between CK2α and Phu.

Initial comparison of the predicted amino acid sequence of Phu against a "BLAST" database search (28), identified that its carboxy terminus was identical to the predicted amino acid sequence of a protein identified as an interaction partner for the leucine zipper region of the c-Jun transcription factor (29). Chevray and Nathans,(1992), outlined the existence of a possible leucine zipper within this protein. Given the knowledge that CK2 can phosphorylate transcription factors (4), and that either CK2α or CK2α'

can interact with the basic leucine zipper regions of the c-Jun, c-Fos, ATF-1 and CREB transcription factors (30,31), suggested that Phu may be a factor involved in transcription. Further analysis of the deduced amino acid sequence of Phu led to the identification of a Pleckstrin Homology (PH) domain at its amino-terminus (32). The PH domain of certain proteins has been shown to be a key domain in targeting certain proteins to the plasma membrane via interaction with phosphoinositides (33). These observations, along with recent reports that CK2 has been identified in highly purified plasma membranes (34), and that CK2 associates with the cell surface receptor CD5 (23), suggests that we have identified a protein which associates with CK2α and may recruit it to the plasma membrane.

In an effort to reconcile the seemingly contradictory observations that Phu possesses domains which are consistent with both nuclear and cytoplasmic/plasma membrane functions, we expressed a Enhanced Green Fluorescence Protein (EGFP)-Phu fusion protein in monkey kidney (Cos 7) cells. The EGFP-Phu fusion protein displayed a predominantly cytoplasmic localization, with indications of localization to plasma membrane structures such as membrane ruffles. Meanwhile, EGFP by itself, displayed a general cellular, with a prominently nuclear, localization. The results of our EGFP-Phu localization experiments are strikingly similar to results obtained by Ma et al., (1997), who showed that the

N-terminal PH domain of pleckstrin associated with peripheral membrane ruffles and dorsal membrane projections of Cos 1 cells (35). Altogether, these observations along with our results support the idea that Phu possesses a PH domain which can target it to the plasma membrane. To confirm the plasma membrane localization of Phu, a more detailed microscopic analysis, such as confocal microscopy and labeling of cell surface glycoproteins with fluorescently labeled wheat germ agglutinin (WGA), are required to specifically ascertain the plasma membrane location of Phu. Furthermore, immunohistochemistry using existing Phu and CK2 antibodies, should enable us to examine the cellular localization of Phu, and aid us to determine whether endogenous CK2 and Phu co-localize in cells.

Overall, we have identified a novel protein with the apparent capacity to sequester CK2α to the cytoplasm/plasma membrane perhaps allowing CK2 to respond to plasma membrane receptor signals, and participate in a variety of cytoplasmic and/or plasma membrane functions.

Implications and Future Directions-

The observations that Phu displays a cytoplasmic/plasma membrane localization and contains a PH domain at its amino-terminus, and a putative leucine zipper at its carboxy-terminus are quite intriguing. There are examples of other

proteins which contain both PH domains and leucine zippers, some of which have been reported to interact with members of the Rho GTPase family. These GTPases are cytoplasmic proteins that respond to activation of plasma membranes, and have been implicated in: controlling the organization of the actin cytoskeleton, progression through the G_1, and M phases of the cell cycle, as well as endocytosis and exocytosis (36a, 36b). Considering that CK2 plays a key role in the actin directed polarized growth in yeast (37,38) and in neuritogenesis of mouse neuroblastoma cells (39), is important for cell cycle progression (40), and has been shown to play a role in intracellular trafficking (41), it is compelling to speculate that Phu and CK2α associate in a complex which possesses similar properties to that of certain GTPases associated proteins (i.e. PH domain, kinase activity, proline rich domain, and leucine zipper).

A recent report by Falasca et al., (1998), demonstrated that serum stimulation of serum deprived cells caused a quick and transient cellular re-distribution of the PH domain of PLCγ from the cytoplasm to the plasma membrane (42). This effect was dependent on PI-3-kinase activity, and the production of the specific PLCγ ligand (PtdIns(3,4,5)P_3. Similar experiments should be considered for Phu to determine whether its localization is affected in response to growth factors and/or mitogens. In the same vein, earlier

reports have demonstrated that CK2 activity responds to certain extra-cellular stimuli such as insulin. However, these observations are controversial, and have not been universally observed in all cell lines or laboratories (4,43). In any case, studies, aimed at elucidating a possible relation between CK2 and insulin signaling, have identified key components of the insulin signaling pathway, such as the insulin receptor and IRS-1, as substrates for CK2. Interestingly, both the insulin receptor and IRS-1 have been localized to caveolae, along with other CK2 substrates such as dynamin and caveolin (44,45,46,47). If we consider that Phu associates with and recruits CK2α to the plasma membrane, one may further speculate that Phu recruits CK2α to a specific plasma membrane area, such as caveolae, and allows CK2 to phosphorylate substrates such as caveolin, dynamin, I.R., and IRS-1. This scenario would permit CK2 to participate in such plasma membrane associated processes such as insulin signaling and/or endocytosis. With this in mind, we should examine the possible co-localization of Phu/CK2α with potential plasma membrane association targets, such as caveolae, or specific cell surface receptors. Identification of target areas and/or associated proteins will undoubtedly enable us to elucidate the function of the CK2α /Phu complex in cells.

The exact nature of the Phu/CK2α interaction needs to be

studied. We are in a position to perform straight forward experiments to determine whether the entire CK2 holoenzyme can associate with Phu, or whether it is an exclusively a Phu/CK2α interaction. We can also determine whether Phu interferes with interactions between CK2α and CK2β. Moreover, we should be able to identify the domains of interaction between Phu and CK2α. With this information, it may be possible to disrupt interactions between CK2 and Phu in order to examine the functional consequences of the interaction.

Preliminary experiments have failed to demonstrate any significant phosphorylation of GST-Phu using purified CK2 *in vitro*. We do not know whether this is a true reflection of the phosphorylation state of Phu in cells, or whether the lack of phosphorylation is related to the conditions used for the assays. Furthermore, although we have no evidence that GST-Phu can significantly alter CK2 activity *in vitro*, we cannot exclude such a phenomenon in cells. I believe that attempts to obtain enriched and/or purified fractions of Phu from cell extracts should lead to a better resolution of these issues. Moreover, biochemical fractionation or co-immunoprecipitations may lead to the identification of proteins which associate with Phu or Phu/CK2α.

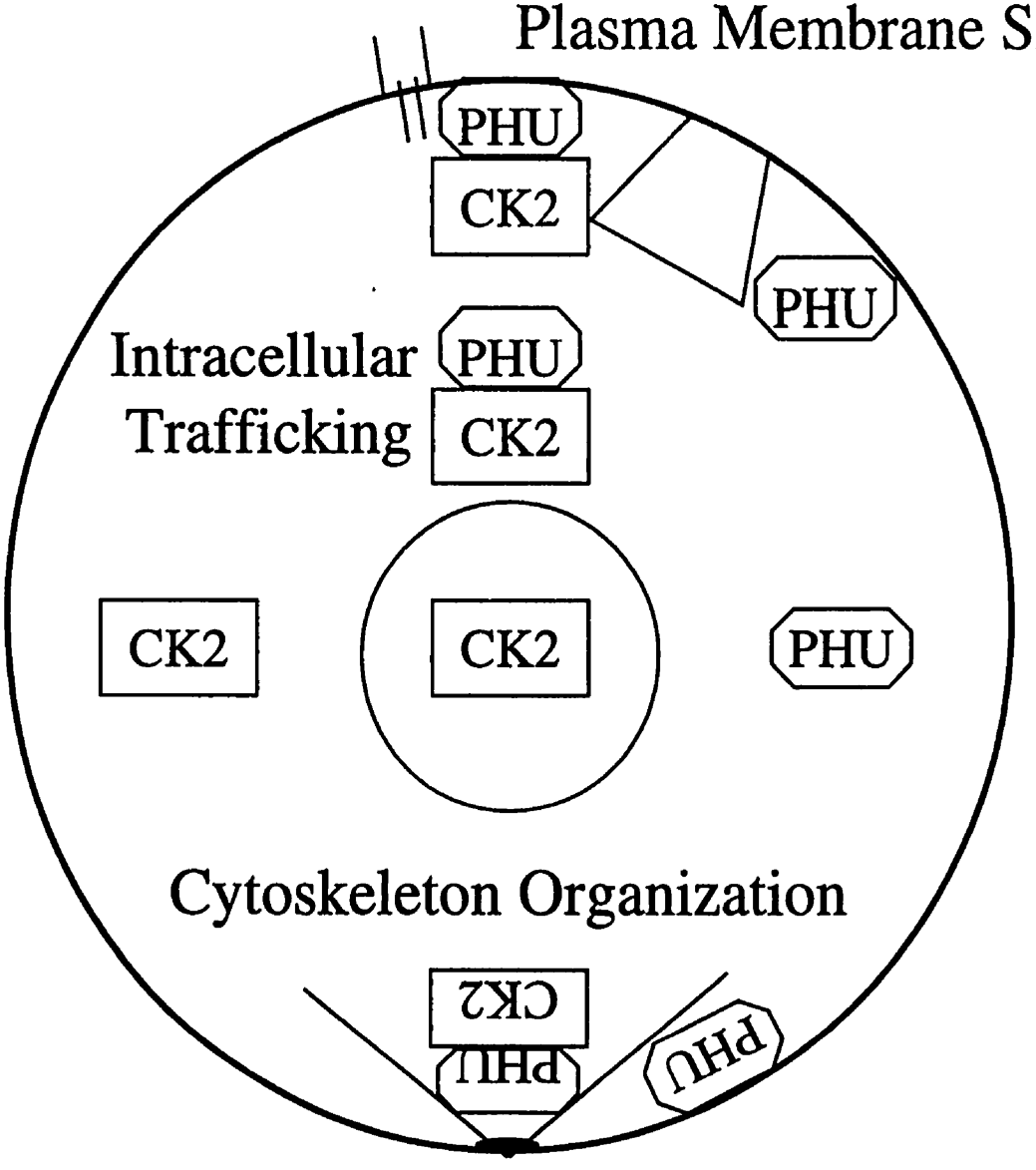
The observations that the Phu CK2α interaction is

mediated through the CTD of CK2α, and that the CTD of CK2α is phosphorylated in mitosis, intuitively one may suspect that the Phu/CK2α interaction may be affected by phosphorylation. In fact, the mitotic phosphorylation of CK2α-CTD coupled with the interaction of Phu with CK2α-CTD suggests that Phu/CK2α interaction may be regulated in a cell cycle dependent manner. Furthermore, since the CTD of CK2α is the target of mitotic phosphorylation and also important for interaction with Phu, an obvious thing to test is whether the phosphorylation of CK2α at its CTD affects its interaction with Phu. Furthermore, since the CTD of CK2α is phosphorylated during mitosis, it is plausible to postulate that Phu may also be a target of $p34^{cdc2}$, especially considering that Phu contains some canonical $p34^{cdc2}$ phosphorylation sites.

The observation that the Phu protein is not detected equally in all cell lines examined, suggest that Phu may have cell type specific functions. We were able to detect significant levels of Phu in Western blot analysis of human osteosarcoma (Saos-2) cell extracts, indicating a possible role for Phu in bone physiology. There have been observations that CK2 phosphorylates certain key bone-phosphoproteins, such as osteopontin, which are essential for bone mineralization and function (48,49,50). Meanwhile,

Northern blot analysis also suggests that Phu expression is not uniform in all human tissues. We detected a higher expression level of Phu message in skeletal muscle and heart tissues, which could indicate a specialized function for Phu in muscle physiology. In support of this idea is that CK2 has been shown to positively regulate myogenesis through phosphorylation of the E47 transcriptional inhibitor (51).

Fig. 5.2 **Cellular localization and function of Phu.** Schematic representation of a cell which depicts the possible localization of Phu, and some of the cellular processes in which Phu, by itself or associated with CK2, may participate.


Plasma Membrane Signaling
PHU
CK2
PHU
Intracellular
Trafficking
PHU
CK2
CK2
CK2
PHU
Cytoskeleton Organization
CK2
PHU
PHU

CONCLUSION

Our cell cycle dependent studies of CK2 have allowed us to determine that there is no relative differences in expression of CK2α versus CK2α' through cell cycle progression. However, we did observe a marked decrease in protein levels of both CK2α and CK2α' in quiescent cells compared to proliferating cells. We also observed an increase in protein levels of CK2β in mitotically arrested cells. These results suggest that expression of CK2 subunits may regulate the entry and exit of cells in the cell cycle.

The protein interaction studies have allowed us to identify a novel protein, Phu, which displays isoform specificity in that it interacts with CK2α, but not with CK2α', and that this interaction is mediated through the unique CTD of CK2α. This finding supports our hypothesis that CK2α and CK2α' exhibit functional specialization in cells. Furthermore, the identification that Phu is targeted to the cytoplasm/plasma membrane indicates that we may have uncovered a new level of regulation for CK2 by affecting its cellular localization.

In conclusion, it is apparent that the pleiotropic nature and complex regulation of CK2 in cells make this an

interesting and important enzyme to study. Our laboratory was able to make some key observations which will undoubtedly lead to a better understanding of the regulation of CK2 in mammalian cells, and help to clarify the role of CK2 in cell growth and proliferation.

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IMAGE EVALUATION
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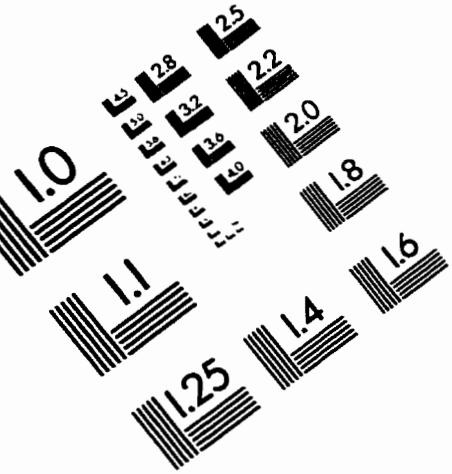

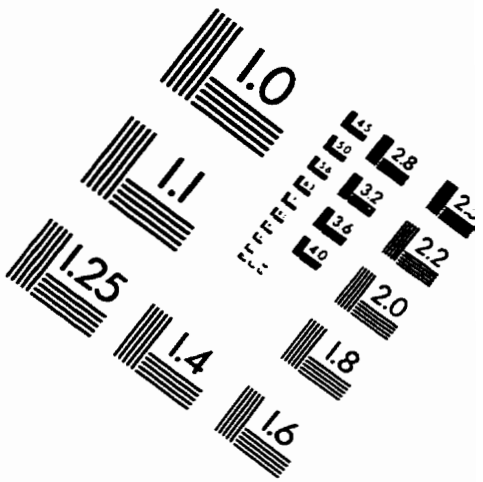

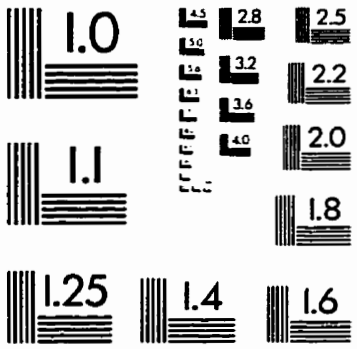

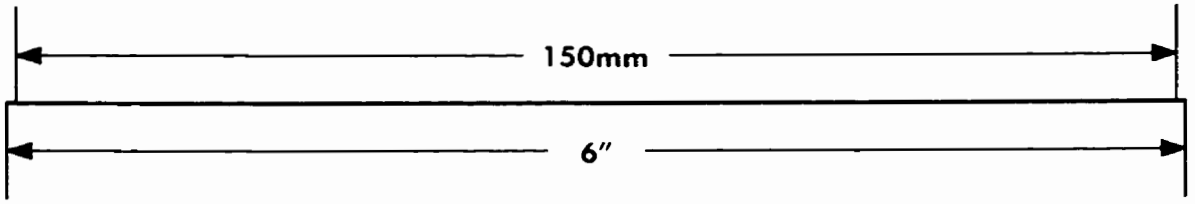

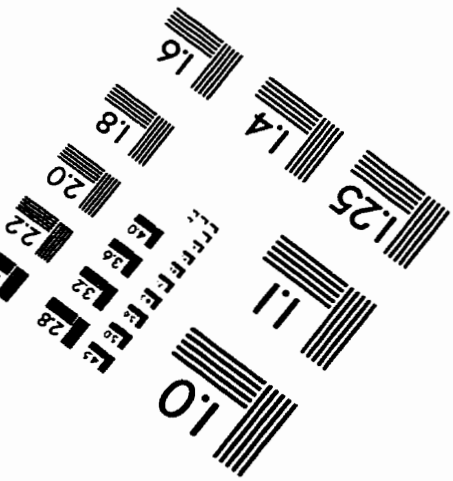

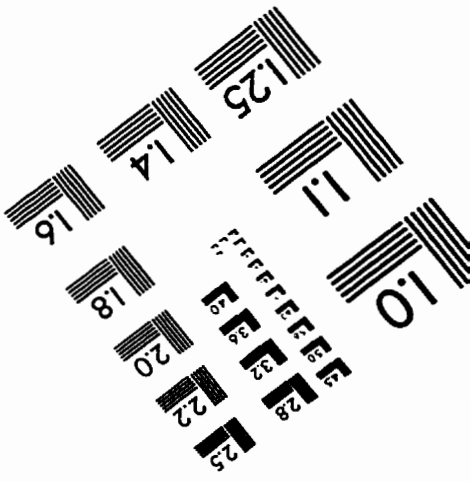

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