REGULATION AND FUNCTION OF PROTEIN KINASE CK2 IN CANCER CELLS

By

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ABSTRACT

Protein kinase CK2 is a ubiquitous eukaryotic protein serine/threonine protein kinase present in both the nucleus and cytoplasm. In most cells, the holoenzyme form of CK2 is a constitutively active heterotetramer of two catalytic (α and α') and two non-catalytic β-subunits, but CK2β-free pools of CK2α also exist. The activity of CK2 is increased in rapidly growing tissues, tumor cells and human cancers. Despite the identification of more than 160 substrates, many of which are important in cell division and differentiation, the signaling pathways that regulate this enzyme have been enigmatic. Here I provide evidence that protein kinase CK2 is regulated by the stress-activated p38 MAP kinase signaling pathway. I found that p38 MAP kinase, in a phosphorylation-dependent manner, can directly interact with and activate CK2 apparently through an allosteric mechanism.

The tumor suppressor protein p53 is one of the key molecules involved in maintaining the stability of the genome, through both cell cycle arrest and apoptosis, especially under circumstances of genotoxic stress. p53 has previously been shown to be associated with protein kinase CK2, and undergo phosphorylation on Ser-392. However, the physiological regulation and the functional consequences of this event remain unknown. I found that this phosphorylation is dependent upon the stepwise activation of p38 MAP kinase and CK2 in response to cellular stress. Furthermore, this activation was demonstrated to be responsible for the biochemical regulation of the cyclin-dependent kinase-1 (CDK-1), as both 5,6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole (DRB; a semi-specific inhibitor of CK2)) and antisense depletion of CK2, as well as SB203580 (a

specific inhibitor of p38 MAP kinase) were each associated with an inhibition of its activation in response to nocodazole (microtubule inhibitor). I observed that depletion or inhibition of the catalytic subunits of CK2, in the presence of microtubule inhibitor (nocodazole), resulted in a compromise of the spindle assembly checkpoint. Furthermore, the CK2-depleted cells exposed to nocodazole underwent reduced apoptosis as well as abnormal duplication and alignment of centrosomes. These oncogenic properties were observed in both human cervical carcinoma cells (i.e. HeLa cells) and human colon tumor cells (HCT116). I also showed that this effect was dependent on the presence of functional wild-type p53, as this phenomenon was not apparent in HCT116 $p53^{-/-}$ cells. My results support a novel role for CK2 in concert with p53, to maintain the stability of the genome. These findings may provide for an improved understanding of abnormalities of the spindle checkpoint in human cancers, which is a prerequisite for defining future therapies.

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LIST OF ABBREVIATONS

APC	Anaphase-promoting complex
AS	Antisense
ATM	Ataxia telangiectasia mutated
CAK	Cyclin-activating kinase
CK2	Casein kinase II
CDK	Cyclin-dependent kinase
BAX	Bcl2 associated factor X
Caspase	Cysteine-aspartic acid specific protease
DNA-PK	DNA-dependent protein kinase
DRB	5,6-dichloro-1-(β- D-ribofuranosyl)-benzimidazole
EGF	Epidermal growth factor
EDTA	Ethylene diaminetetra-acetate
Erk-1	Extracellular regulated kinase-1
Erk-2	Extracellular regulated kinase-2
FGF	Fibroblast growth factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK-3	Glycogen synthase kinase-3
HSP27	27-kDa heat shock protein
IC	Inhibitory concentration
IGF-1	Insulin-like growth factor-1
IGF-BP3	Insulin growth factor-binding protein-3
IL	Interleukin

JNK	c-jun N-terminal kinase
kDa	KiloDalton
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MAPKAP	MAPK-activated protein
Mr	Relative molecular mass
MW	Molecular weight
MEF	Mouse embryonic fibroblast
MKK	MAP kinase kinase
Mdm2	Murine double minute clone 2
MPF	Maturation promoting factor
NGF	Nerve growth factor
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophoresis
p38 MAP kinase	38 kDa mitogen-activated protein kinase
p53	53 kDa tumor suppressor protein
PBP	Polybasic peptide
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PIGs	p53-induced genes
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatase 2A

RB	Retinoblastoma
RGS	Ribosomal gene cluster
S	Sense
SB203580	SmithKline Beecham-203580; p38 MAPK inhibitor
SDS	Sodium dodecyl sulphate
SH3	Src-homology domain-3
TAF	TATA box binding protein associated factor
TBP	TATA-binding protein
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

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1. INTRODUCTION

1.1. Protein Kinase CK2

1.1.1. Generalities

Protein kinase CK2 (formerly termed casein kinase II) is a highly conserved protein-serine/threonine kinase that is ubiquitously expressed in eukaryotic organisms. CK2 from most sources is generally a heterotetramer with a molecular mass of approximately 130 kDa. This tetramer is composed of two subunits with a general stucture of $\alpha 2\ \beta 2$ or $\alpha\alpha'\ \beta 2$. The α and α' are catalytically active by themselves and have molecular masses of 42-44 kDa (α) and 38 kDa (α'). The α and α' subunits are structurally analogous but are known to be encoded by different genes (Litchfield et al., 1990). Although the α subunits are found in all species, the α' subunits have not been found in *Drosophila* and *Xenopus* (Allende and Allende, 1995).

The β subunit exists in homodimers (usually 26 kDa in all animal cells) and is inactive, but confers both negative and positive regulation to the catalytic activity of α subunits (Pinna, 1990; Issinger, 1993). In addition, the β subunit affects the stabilization of α subunits against heat denaturation or proteolysis and, more importantly, it can change the specificity of its interaction with substrates and inhibitors (Allende and Allende, 1995; Chambaz, 1998). In *Saccaromyces cerevisiae* two forms of the β subunit exist, β and β' (41 and 32 kDa), which are expressed by two separate genes (Bidwai et al., 1994). In *Dictostelium discoideum* and *Zea mays* there are no detectable β subunits,

despite the existence of intact CK2 catalytic activity (Allende and Allende, 1995; Guerra et al., 1999a).

The α-subunit shares a high degree of homology with cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases) and glycogen synthase kinase-3 (GSK-3), whereas the non-catalytic β-subunit is a relatively unique protein with no remarkable homology to other proteins (Pinna and Meggio, 1997; NCBI Genbank, 2001). The tetrameric holoenzyme is extremely stable. *In vitro*, the holoenzyme is formed upon mixing of the subunits by a self-assembly mechanism, and cannot be easily dissociated, except under denaturing conditions (Grankowski et al., 1991). Whether dissociation occurs *in vivo* once the holoenzyme has been formed is totally unknown.

The determination of the crystal structure of the catalytic subunit of protein kinase CK2 from *Zea mays* (recombinant maize CK2α) at 2.1 Å resolution was fundamentally important, as it sheds light on some of the biochemical characteristics of this enzyme (Guerra et al., 1998; Niefind et al., 1998). CK2 is unusual among protein kinases in that it can use both ATP and GTP as phosphoryl donors with almost the same efficiency. Its heterotetrameric structure is rare among protein kinases and it has a predilection for phosphoacceptor residues having clusters of acidic amino acids on their C-terminal side. Such remarkable site specificity is dictated by a minimum consensus sequence, S/T-X-X-E/D/Sp/T (Tuazon and Traugh, 1991; Pinna and Meggio, 1997).

Although CK2 was one of the first protein kinases discovered (Burnett and Kennedy, 1954; Pinna, 1990; Issinger, 1993; Allende and Allende, 1995; Pinna, 1997; Guerra and Issinger, 1999), its regulation by known second-messenger molecules and its physiological function have been elusive. Numerous reports indicate that CK2 is

involved in proliferation and tumorigenesis. CK2 phosphotransferase activity has been shown to be enhanced in transformed cell lines (Prowald et al., 1984, Chambaz, 1998), in solid tumors (Munstermann et al., 1990; Pistorius et al., 1991; Stalter et al., 1994; Landesman-Bollag et al., 2001), in rapidly proliferating tissue (Munstermann et al., 1990; Lebrin et al., 2001), and also during embryogenesis (Schneider et al., 1986; Mestres et al., 1994). In higher eukaryotic organisms, prominent CK2 activity has been found in embryonic brain, indicating an important role for CK2 in brain development (Mestres et al., 1994). It has been shown that embryonic tissues or organs contain higher concentrations of CK2 protein level and activity than in the adult counterparts over a wide range of species that include rat, chicken and the nematode *C. elegans* (Issinger, 1993; Allende and Allende, 1995). Studies using in situ hybridization for detection of mRNAs in mice and in zebrafish during embryonic development have demonstrated that mRNAs for the α and β subunits of CK2 are especially abundant in the developing nervous system (Mestres et al., 1994). These findings correlate nicely with the observation that microinjection of antisense oligonucleotides directed against CK2α prevents neuritogenesis in neuroblastoma cells (Ulloa et al., 1993).

In solid human tumors, e.g. mammary, colorectal and lung carcinomas, CK2 amount/activity is abnormally elevated (Issinger, 1993; Daya-Makin et al., 1994; Gapany et al., 1995; Faust et al., 1996; Guerra et al., 1999a; Landesman-Bollag et al., 2001; Romieu-Mourez et al., 2001). On the one hand, this elevated activity of CK2 has been suggested by Seldin and Leder (1995) to contribute to the pathogenesis of human cancer. On the other hand, CK2 can also negatively regulate cell proliferation (Allende and Allende, 1995; Pinna, 1997 Guerra et al., 1999a). It has been shown that CK2 may exert

its anti-proliferative activity by its interaction with the tumor suppressor protein p53 (Milne et al., 1992), or with protein phosphatase 2A (Heriche et al., 1997).

Recent studies indicate that CK2 is not only active as a tetrameric holoenzyme but also as free subunits (Allende and Allende, 1995; Xin et al., 1999; Landsman-Bollag 2001). Stigare et al. (1993) have reported that the majority of the CK2 α-subunit is tightly bound to intranuclear components other than the β-subunit. The regulatory β-subunit is produced far in excess of the α–subunit and is rapidly degraded (Guerra et al., 1999b). In cancer cells an abnormal distribution and ratio of α- and β- subunits was observed, and this dysregulation has been proposed to contribute to the malignant phenotype (Guerra et al., 1999b). Overexpression of CK2 has been studied in mice. It has been shown that transgenic mice, expressing elevated levels of CK2 α in lymphoid organs, display a stochastic propensity to develop lymphomas (Seldin and Leder, 1995). Co-expression of a c-myc transgene in addition to CK2 α results in neonatal leukemia, indicating that CK2 α can serve as an oncogene and that its dysregulated expression is capable of transforming lymphocytes (Seldin and Leder, 1995). Furthermore, it has been reported that CK2α overexpression is involved in the loss of a p53 allele in thymic lymphomas (Ole-Moi et al., 1993). The role of CK2 in cancer is discussed below.

Clues regarding the function of CK2 *in vivo* have also been obtained via identification of putative physiological substrates and by molecular genetic studies. Over 160 well characterized substrates of CK2 are currently known (Pinna, 1997; Pinna and Meggio, 1997). The alphabetical list of proteins phosphorylated by protein kinase CK2 is shown in Table 1. Among them are nuclear and cytoplasmic enzymes and structural proteins playing important roles in transcription, translation, signal transduction and cell-

cycle regulation. Disruption of CK2 α-subunit genes in yeast revealed that the catalytic CK2 α-subunit is essential for viability (Padmanabha et al., 1990), whereas overexpression did not lead to detectable phenotypic changes (Roussou and Draetta, 1994). In contrast, yeast strains lacking CK2 β are viable, but exhibit an altered phenotype, and overexpression of CK2 β also leads to an altered phenotype (Roussou and Draetta, 1994; Bidwai et al., 1995). Recent research indicates that CK2 subunits could be involved not only in forming the tetrameric CK2 holoenzyme, but also in binding to, and possibly regulating, other proteins such as transcription factors (e.g. p53 protein and TAL-1) and protein kinases, (e.g. A-Raf kinase and Mos) (Boldyreff and Issinger, 1997; Hagemann et al., 1997; Chen, et al., 1997; Xin et al., 1999). This seems to be especially interesting in light of the finding that CK2 β/α-subunit distribution is unbalanced in clear cell renal carcinomas, with an excess of β-subunits in the carcinomas (Stalter et al., 1994).

Table 1. Alphabetical list of proteins phosphorylated and interacting with protein kinase CK2 (Pinna and Meggio, 1997)

Al (human hnRNP)
ABF-1 (yeast)
Adenovirus Ela
Androgen receptor
c-jun
c-Myb
C-Myc
Ca^{2+} channel blockers; receptor
Caldesmon
Calmodulin
Calnexin
Calreticulin (spinach)
Calsequestrin
Caveolin
CDC37
CD45 tyrosine phosphatase
Clathrin light β-chain
Coactivator p15 (PC4)
CREB
CREM
CTP:phosphocol. cytidyltransferase
DARPP-32
DNA ligase
DNA topoisomerase I
DNA topoisomerase Il
DSIP peptide
Dynamin
Dystrophin
E47
EF-1 6
EF-1 P
Egr-1 (early growth response)
eIF-2a
eIF-2p
eIF-3
eIF-4B
eIF-5
eIF4e/p2O (*S. cerevisiae*)
Engrailed protein *(Drosophila)*
Epstein-Barr Nuclear Antigen 2
Epstein-Barr SM, P-protein
Epstein-Barr virus ZEBRA protein
Furin
Cap43 (neuromodulin)
GB glycoprotein (cytomegalovirus)
GBF-1 transcription factor (Arabidopsis)
Glycogen synthase
Glycophorin
gp96 (soybeans). .
CRP94 of sarcoplasmic reticulum'
Guanine nucleotide exchange factor
HASPP28 (rat brain)
HCP (hist.-rich Ca^{2+} bind. protein)
HDAgs (Hepatitis 8 virus antigens)
HIV-1 Rev protein
HrV-1 viral protein
HMG -14
HMG -1
HMG-like protein P1
hnRNP
HPV E7
Hsp 9O
Human centrom. protein 8 (CENP-B)
Human complement protease
Human estrogen receptor
IGF-11 receptor
IGFBP-1
IkBα
IkBβ
Inhibitor-2 of PP1
Insulin receptor
IRS-1
LD lipoprotein receptor
Upoprotein inhibitor
Mannose-6-phosphate receptor
MAP-1B
Max protein
MDM2
Measles virus P-protein
MEF2C (myocyte enhancer factor)
MRFs (MRF4 and MyoD)
mRNA particles
MYCN (human neuroblastoma cell)

Table 1. Continued

NBP-60 (NLS binding protein)
Neural cell adhesion molecule
Nuclear DNA-binding protein p210
Nucleolar p130
Nucleolar protein B23
Nucleolin
Nucleoplasmin
Ornithine decarboxylase
Osteopontin
P0, P1, and P2 ribosomal proteins
p120 cell prolif. nucleolar protein
p13 and p38 yeast ribosomal proteins
p25 (*Xenopus laevis*)
p34 (chloroplast ribonucleoprotein)
P34 ^{cdc2}
p38 RNA-binding protein
p53
p65 synaptic vesicles
p98 (sea urchin egg)
Pea lamin like protein
PHAS-1
PKA R-11 subunit
FKC
Platelet Va and Vllla factors
PP2C
pp35 proliferation nuclear P-protein
pp65 (human cytomegalovirus)
Progesterone receptor
Proteasome (30 kDa)
Proteasome complex (C8 and C9)
Prothymosin α
Pseudorabies virus pp-62
PU-1 transcription factor
RAS-17
Rat epididymal fat cell p22
Respiratory syncytial virus P-protein
RIPP
RIPP-1 and -11 phosphophoryns
RNA polymerase I
RNA polymerase 11
RNA polymerase III
Sarcalumenin
Serum response factor
Stathmin
SV40 large T antigen
Synaptotagmin
T-5ubstrate (wheat germ)
Tal-1
Tau protein
TFILA coactivator p15
TRα2 (thyroid hormone receptor).
Troponin-T
Tryptophanyl-tRNA synthetase
Tubulin
VAMP/synaptobrevin
Viral P-protein
Vitamin D receptor
VPI (polyoma virus capsid)
VPU (HIV-1)
VSV (ves. stomatitis virus) P-protein
VZV Waricella zoster virus) protein
XFC-5-1 (Xenopus zinc finger) protein
xNoppl8O (*Xenopus laevis*)
Yeast SRP-40 (homologue to Nopp140),
ZFPs (Xenopus laevis)

1.1.2. Molecular basis of CK2 specificity

To gain information about the structural elements responsible for the unique ability of CK2 to recognize phospho acceptor sites specified by multiple acidic residues, a systematic mutational study has been performed in which a number of basic residues conserved in CK2α were replaced by alanine. By biochemical analysis of each of these mutants with the optimal substrate RRRADDSDDDDD, a number of basic residues within the α subunit that have been implicated in substrate recognition, have been identified (Sarno et al., 1996; 1997a). By combining this information with modeling studies, a sequence of basic residues concentrated around the catalytic site, and responsible for the binding of individual acidic determinants in the phosphoacceptor substrate with a minimum consensus sequence, S/T-X-X-E/D/Sp/Tp has been highlighted, where X is often an acidic amino acid residue (Sarno et al., 1997a, 1998). These authors have shown that the main contacts are those between the aspartic acid residue at position n+1 and n+3 with Lys-198 and one or more of the lysines in the ^{74}KKKK^{77} quartet located at the basic stretch between the conserved regions II and III (Sarno et al., 1997a, 1998). Additional contacts involve two basic residues that are inserted in the Gly-rich loop and possibly a lysyl residue in the subdomain V region (Lys-122) (Vaglio et al., 1996).

Sarno et al. (1996; 1997b) have shown that by mutating basic residues in the 74-77 and in the 191-198 regions of the alpha-subunit, the negative regulation by the beta-subunit and by its N-terminal synthetic fragment CK2β-(1-77) is drastically reduced. In contrast, the positive regulation by a C-terminal, CK2β-(155-215)-peptide is unaffected or even increased. Moreover, the basal activity of the α subunit mutants K74-77A,

K79R80K83A, and R191R195K198A towards specific peptide substrates is stimulated by the β subunit many fold more than that of alpha wild type, while extra stimulation by β mutant D55L56E57A, observable with α wild type, is abolished with these mutants (Sarno et al., 1996; 1997a). These data support the conclusion that down regulation by the acidic residues clustered in the N-terminal moiety of the β subunit, is mediated by basic residues in the 74-83 and in the 191-198 sequences of the α subunit. These are also implicated in substrate recognition, consistent with the concept that the N-terminal acidic region of the β subunit operates as a pseudosubstrate (Sarno et al., 1997b). The structural analysis of CK2 is discussed below. A schematic illustration of CK2 residues implicated in substrate recognition and accounting for the markedly acidophilic nature of CK2 is shown in Figure 1.

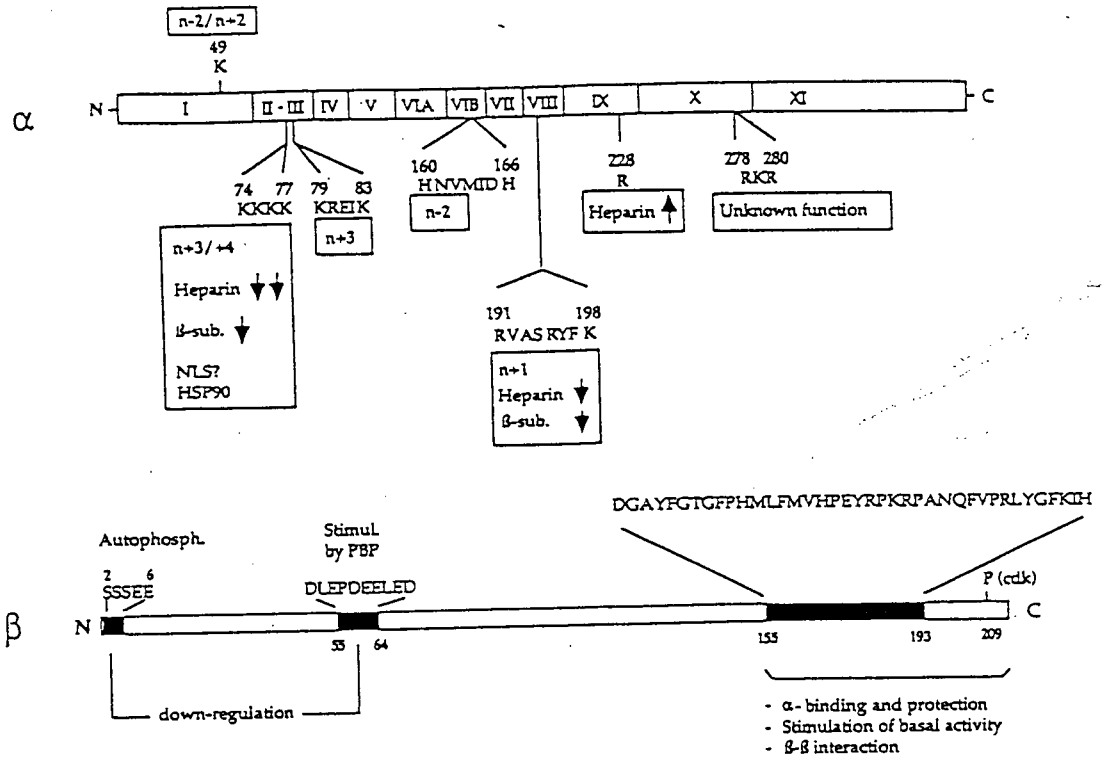

Figure-1: Functional elements of CK2 α and β subunits. Regions and/or residues implicated in specific functions of α and β subunits are highlighted. The Roman numerals denote the sub-domains of the CK2α catalytic domains as defined by Hanks and Hunter (1995). The positions (relative to Ser/Thr) of the substrate specificity determinants recognized by basic residues of the α subunit are specified by the n+/n- notation. Arrows pointing up and down indicate stimulation and inhibition by heparin (or β subunit), respectively. NLS, nuclear localization signal; PBP, polybasic peptides (e.g. polylysine, histones); HSP 90, heat shock protein-90. Based on data from Pinna and Meggio (1997) and Pinna (1997).

1.1.3. Structural Features

1.1.3.1. The catalytic subuits α/α'

The primary structure of the CK2 catalytic subunits (either α or α') displays very highly conserved regions of the 12 subdomains that have become the hallmarks of the large superfamily of protein kinases (Litchfield et al., 1990). I will discuss the most important and well characterized domains of CK2α.

Conserved region I (glycine loop)

As in all kinases this region is involved in ATP binding and phosphotransfer. The first two glycines out of the three of the motif G-X-G-X-X-G are conserved (Pinna and Meggio, 1997). The third residue in CK2 α is replaced by a hydrophilic residue, serine, a feature that might confer ATPase activity by bridging water molecules in contact with ATP (Pinna and Meggio, 1997). Two features of this loop are notable: a lysine residue (K49) which has been shown by mutational analysis to contribute to the recognition of the acidic determinant at position n+2 in peptide substrates (Sarno et al., 1998); and a tyrosine (Tyr-50) which is homologous to the regulatory residue Tyr-15 of CDK, the phosphorylation of which (as well as that of the preceding Thr-14) suppresses the catalytic activity of CDK. The question of whether this tyrosine residue is ever phosphorylated in CK2 as a mechanism for its regulation remains unclear.

Basic stretch between conserved regions II and III

This region comprises amino acids 74-80 which is conserved in all species and probably is the most striking hallmark of CK2 and is situated just upstream from the conserved glutamic acid that defines subdomain III. It is involved in substrate recognition (position n+3; Fig. 1) and interaction with the β-subunit.

Mutational analyses have shown that the first part of this cluster (Lys-74 to Lys-77) (Hu and Rubin, 1990; Gatica et al., 1994; Vaglio et al., 1996), is implicated in CK2 inhibition by heparin. However, both the 74-77 and the 79-83 segments are implicated in substrate recognition at position n+3 (Guerra et al., 1999a). Although it appears that these basic residues are to some extent interchangeable as far as substrate recognition is concerned, Lys-77 seemingly plays a prominent role since individual replacement by alanine causes a dramatic increase of the phosphotransferase activity of the α subunits toward casein (Sarno et al., 1998).

It has been shown that the 74-80 basic sequence of CK2α interacts with the β-subunit (Sarno et al., 1998) and mediates the β subunit induced down regulation of the catalytic activity of CK2 (Pinna and Meggio, 1997). Competition experiments with heparin have revealed that the 74-77 basic quartet also mediates interactions with HSP90 (Miyata and Yahara, 1995). This basic region, in addition, has the hallmarks of a nuclear localization signal (NLS), which may be responsible for targeting CK2 to the cell nucleus (Rihs et al., 1991). These studies have been confirmed by the crystal structure of recombinant maize (rm) CK2 α (Niefind et al., 1998; Guerra et al., 1999a). The

structure of rm CK2α closely resembles the well-known structure of protein kinase A (PKA), with a β-rich N-terminal domain comprising the β strands 1–5 and the helix αC. The CK2 enzyme was crystallized in an active, open conformation where the N-terminal region, characterized by short α-helical motifs, forms extensive contacts with the lysine-rich cluster of the αC helix and with the activation loop. The lysine-rich cluster is characterized by the K^{74}KKK-IKREIK^{83} sequence. This cluster is involved in substrate recognition, inhibition by heparin, down-regulation exerted by the CK2β subunit, interaction with heat shock protein-90, and nuclear targeting mediated by a nuclear localization signal (Nigg et al., 1991, Niefind et al., 1998).

The catalytic loop and PP2A binding motif

This short segment contains the D X^1 K X^2 X^3 N sequence, spanning the highly conserved Asp and Asn residues that define subdomain VIB in CK2 (Figure 1) and is almost identical to PKA and CDKs. This region is important in peptide substrate recognition in PKA. Mutational studies in this region of CK2 were indicative of the acidophilic nature of this kinase (Dobrowolska et al., 1994). Just downstream from the catalytic loop is a sequence motif conserved in vertebrate and *Drosophila* CK2α (H-F-H/N-R-K-L). This motif is also found in SV40 and is required for binding protein phosphatase 2A *in vitro* (Heriche et al., 1997).

The subdomain VII-VIII connection (activation loop)

The region between the DFG and APE triplets that defines subdomains VII and VIII in most protein kinases is altered in CK2 (Guerra et al., 1999a). This region contains the 'activation loop' (or 'T-loop') after the presence of a threonyl residue that is constitutively phosphorylated in active PKA (Pinna and Meggio, 1997). The activation loop includes residue(s) that when phosphorylated (either autocatalytic or promoted by other kinases) correlate directly with increases in the enzymatic activity of most kinases (Johnson et al., 1996). However, CK2α does not contain either threonine or serine in its activation loop, nor has phosphorylation ever been reported in this region. These observations, in conjunction with the finding that the free catalytic subunit is spontaneously active, indicate that the activation loop of CK2 is displaced from the catalytic cleft and does not hamper enzymatic activity (Pinna and Meggio, 1997).

1.1.3.2. The noncatalytic CK2 β-subunit

The noncatalytic or the regulatory β-subunit of CK2 is generally autophosphorylated *in vivo* and *in vitro*. Peptide mapping studies indicate that the autophosphorylated sites in the β subunit are Ser-2 and Ser-3 (Figure 1). CK2β is a relatively unique protein with no homology to regulatory subunits or domains of other protein kinases. It can be separated from the holoenzyme *in vitro* only under denaturing conditions. After expression of the CK2 subunits α and β separately in *Escherichia coli* and reconstitution of the holoenzyme, it was possible to study the role of the β-subunit by

comparing the features of the α-subunit alone with those of the holoenzyme (Grankowski et al., 1991). The β-subunit has three known functions: (1) it modulates the activity of the α-subunit in a substrate-dependent manner (Grankowski et al., 1991; Meggio et al., 1992b); (2) it contributes to the substrate specificity (Meggio et al., 1992b); and (3) it stabilizes the α-subunit (Issinger et al., 1992; Meggio et al., 1992b). The modulation of the CK2α activity with most substrates by the β subunit is a stimulatory effect. However, if calmodulin is used as the substrate, the β-subunit almost completely inhibits this activity. This inhibition can be overcome by the addition of polylysine (Meggio et al., 1992a). Mutagenesis studies on the β-subunit revealed an acidic stretch from amino acids 55–64, which is responsible for the inhibitory effect and for the stimulation by polylysine (Meggio et al., 1994a). Interestingly, mutations of acidic residues at positions 55, 57, and 59–64 of the β-subunit to alanine residue, led to a 4-fold more active holoenzyme than the wild-type enzyme after reconstitution with the α-subunit alone (Boldyreff et al., 1993). At the same time, these mutants are no longer responsive to the stimulator polylysine. This indicates that the acidic residues Asp-55 and Glu-57 have a general down-regulatory role, which can be overcome by polylysine, most likely by direct interaction with these residues. In the case of the substrate calmodulin, this down-regulation results in almost complete inhibition. Crosslinking and peptide studies have shown that the acidic amino acid stretch of CK2 β from residues 55–64 interacts with a corresponding basic stretch of the α-subunit (Krehan et al., 1996; Sarno et al., 1997b). However, this seems to be a low affinity electrostatic interaction (Guerra et al., 1999a; 1999b).

The structural features that are responsible for β subunit homodimerization, association with the α-subunit, protection against denaturation and proteolysis and up-regulation of the activity, are located in the C-terminal region between amino acids (155-215) (Kusk et al., 1995; Marin et al., 1997). The C-terminal region is also essential for interaction with protein kinases such as the tumor suppressor p53 (Xu et al., 1999), A-Raf (Boldyreff and Issinger, 1997; Hagemann et al., 1997), Mos (Chen et al., 1997), $p90^{rsk}$ (Kusk et al., 1999) and many 'cellular partners' of CK2 (table-1) (Pinna and Meggio, 1997; Kusk et al., 1999).

1.1.4. Regulation

Despite the fact that this enzyme was discovered over 45 years ago (Allende and Allende, 1995; Pinna and Meggio, 1997), the mechanisms that regulate its activity under physiological conditions have been elusive. Here I will discuss some of aspects that have been reported to influence CK2 regulation.

1.1.4.1. The α and β subunit relationship

As mentioned above, two regions of the β-subunit have been shown to interact with the α subunit: the acidic stretch, composed of residues 55–64 in the N-terminal moiety, and the C-terminal region. The N-terminal acidic cluster of β makes electrostatic contacts with basic residues of the α-subunit that are otherwise implicated in substrate binding, and, therefore, is responsible for down-regulation of its activity (Sarno et al.,

1997b). However, the tight physical interaction between the two subunits appears to be mediated by the C-terminal region of the β subunit. The precise location of the interacting domain within the α subunit is unknown. Furthermore, studies exploiting the two-hybrid system in yeast have shown that the entire sequence of the α-subunit, with the exception of only two short segments at the very N- and C-terminal ends, is required in order to ensure strong interaction with the β subunit (Kusk et al., 1995). This outcome is consistent with the view that the correct folding of the α-subunit is required in order to generate the structural elements that bind the β-subunit with high affinity.

1.1.4.2. Activators and inhibitors of CK2

CK2 is unique amongst protein kinases in that it possesses basal activity, either in its canonical heterotetrameric form or as isolated catalytic subunits, being independent of any known second messenger and not susceptible to acute regulation by phosphorylation. This activity is especially intriguing if it is borne in mind that CK2 is implicated in a variety of signal transduction pathways, as judged by the nature of many of its physiological targets. Despite its basal activity, CK2 can still be affected by a number of compounds that have some physiological importance. The list includes compounds such as polyamines, specifically spermine (Allende and Allende, 1995). This compound can cause a two- to three-fold increase in enzyme activity. This stimulation is highly dependent on the concentration of Mg^{2+} and on the nature of the substrate.

It has been reported that a number of polyanions, notably heparin, polyglutamic acid, and random polymers of glutamic acid and tyrosine, are powerful and relatively

selective inhibitors of CK2 (Pinna and Meggio, 1997). Their mode of action is generally competitive with respect to the phosphoacceptor substrate, although heparin does not exactly mimic the substrate conformation when it binds to CK2, as judged from mutational analysis of residues that are implicated in substrate conformation (Sarno et al., 1998). Pseudosubstrate peptides in which serine has been replaced by alanine are poor inhibitors due to a reduction in affinity (Pinna, 1990; Meggio et al., 1994b). In contrast, high affinity is maintained if serine is replaced by tyrosine, consistent with the view that tyrosine can bind, although it is not phosphorylated, except under special circumstances. Consequently, the pseudosubstrate peptide EEEEEYEEEEEEE is a selective and fairly efficient inhibitor of CK2 with an IC_{50} = 0.8 μM (Sarno et al., 1999). It has been shown that protein kinase inhibitors that are competitive with respect to ATP, such as the most potent broad-specific inhibitor of protein kinases, staurosporine, are poor inhibitors of CK2 (Meggio et al., 1995; Pinna and Meggio, 1997; Guerra et al., 1999a). This observation, in conjunction with the ability of CK2 to use GTP, as well as ATP, as a phosphodonor, supports the view that the ATP-binding site of CK2 has unique features that could be exploited to design highly specific inhibitors. This has been confirmed by the resolution of the crystal structure of CK2α (Niefind et al., 1998).

Attempts to improve the selectivity of isoquinoline sulfonamide analog inhibitors of protein kinases have led to the development of a semi-specific inhibitor for CK2 that is derived from the halogenated benzimidazole/benzotriazole derivatives. This compound is 5,6- dichloro-1-(β-D-ribofuranosyl)-benzimidazole, commonly known and commercially available as DRB. DRB, first described as an inhibitor of transcription in eukaryotic systems (Sehgal and Tamm, 1978; Egyhazi et al., 1982) was later found to act through

the inhibition of CK2 activity (Zandomeni et al., 1986). Its K_i values, calculated with CK2 from various sources (Meggio et al., 1990; Szyszka et al., 1995; Guerra et al., 1999), range between 20 and 30 μM. The selectivity of DRB-derived inhibitors is quite remarkable: neither PKA, protein kinase C, pseudorabies virus protein kinase, nor a number of protein-tyrosine kinases are appreciably affected by concentrations that are dramatically inhibitory toward CK2 (Guerra et al., 1999a).

1.1.4.3. Regulation of CK2 activity

Several years ago a series of reports presented evidence for an increase in the activity of CK2 after treatment of animal cells in culture with a number of mitogenic stimuli. These reports included experiments with serum (Carroll and Marshak, 1989), insulin and insulin-like growth factor-IGF-1 (Sommercorn et al., 1987; Klarlund and Czech, 1988;), epidermal growth factor (EGF), heat shock and UV radiation (Sommercorn et al., 1987; Ackerman et al., 1990; Gerber et al., 2000). In general, increases ranging from 30% to four-fold in the level of the activity of the CK2 enzyme were observed 10-30 min after treatment of cells with the stimulating factor. However, these results that appeared to indicate the occurrence of a rapid increase in CK2 phosphotransferase activity after treatment of cells with proliferative stimuli, have been brought into question (Lin et al., 1992; Litchfield et al., 1992). In addition, it has been reported by one of the laboratories that originally described such an increase in the activity, that a systematic study of this phenomenon indicates that the levels of CK2

activity measured *in vitro* after mitogenic stimuli do not significantly change (Allende and Allende, 1995).

Some other observations are also unclear. It has been reported that CK2 phosphotransferase activity is enhanced many fold during progesterone-induced maturation of *Rena temporaria* oocytes (Kandror et al., 1989). By contrast, studying the same phenomenon with *Xenopus laevis* oocytes, Cicirelli et al., (1988) found only a modest 1.7-fold stimulation of CK2, whereas other protein kinases such as RSK, cdk1 and ERK2, increased much more dramatically. Both these observations are in agreement with a report that indicated that *in vitro* the cdc2 kinase [also known as the maturation-promoting factor (MPF) and cyclin-dependent kinase-1 (CDK-1)] obtained from starfish oocytes, could phosphorylate and activate CK2 (Mulner-Lorillon et al., 1990; Smits and Medema, 2001). This report also correlates this activation with the phosphorylation of a threonine residue in the β subunit. Litchfield et al. (1991, 1992) have reported that cdc2 kinase can phosphorylate CK2 *in vivo* and *in vitro*. These authors demonstrated that the phosphorylation occurred on Ser-209 of the β subunit of the mammalian protein. However, they were not able to demonstrate activation of the phosphorylated CK2. Unfortunately, it has been shown that this serine (209) is absent from the β-subunits of *Drosophila*, yeast and *Arabidopis* (Pinna and Meggio, 1997). Furthermore, human CK2β can be deprived of a C-terminal segment without any apparent change in the functional properties of CK2 (Meggio et al., 1993). Similarly, it has been shown that the *in vitro* phosphorylation by the cdc-2 of this site of the β subunit, does not seem to affect the *in vitro* capacity of β to activate the α subunit (Meggio et al., 1993).

Interestingly, it has been also shown that cdc-2 kinase phosphorylates CK2α in cells arrested in mitosis (Litchfield et al., 1992), and conversely that cdc-2 kinase can be phosphorylated at Ser-39 by CK2 during G1 phase (Russo et al., 1992). However, there was no specific effect of this phosphorylation on CK2α activity (Russo et al., 1992). The significance of the relationship between cdc-2 and CK2 remains to be elucidated (Allende and Allende, 1995; Pinna and Meggio, 1997).

1.1.5. CK2 and cancer

The observation that CK2 protein and activity are enhanced in rapidly proliferating tissues dates back 15 years. CK2 protein and enzyme activity appear to be elevated quantitatively in all tumors with proliferation potential (Issinger, 1993; Guerra and Issinger, 1999; Ahmed et al., 2000; Landesman-Bollag et al., 2001).

Increases of CK2 phosphotransferase activity in cancer tissues were first shown in human colonic carcinomas (Seitz et al., 1989; Pistorius et al., 1991). These findings have been confirmed by many other authors, encompassing a large variety of solid tumors, e.g. breast (Munstermann et al., 1990), prostate (Ahmed, 1994), head and neck (Gapany et al., 1995), and lung tumors (Daya-Makin et al., 1994).

Stalter et al. (1994) performed a statistical analysis of the activity and the expression of CK2 using renal clear cell carcinomas and the corresponding control tissues. The average protein kinase CK2 activity from 21 different kidney samples was shown to be 318 U/mg and that from the corresponding tumors, 610 U/mg. The subunit ratio of protein kinase CK2α in tumors/normal tissue (T/N) was 1.58 and that of the

protein kinase CK2β (T/N) was 2.65 (Stalter et al., 1994). The data indicated that the increases in protein kinase CK2 activity in tumor cells may correlate to some extent with a deregulation in subunit biosynthesis or degradation. This is at least partly due to the presence of an excess of enzymatically active protein kinase α- subunit but also to a significantly higher presence of the noncatalytic β-subunit (Stalter et al., 1994).

Caveolin, an integral membrane component of caveolae, was first identified as the major phosphoprotein whose phosphorylation was elevated in v-Src transformed cells. As both v-Src-induced transformation and elevated caveolin phosphorylation were dependent on membrane attachment of v-Src, it has been suggested that caveolin is a critical target in v-Src transformation. CK2 can phosphorylate caveolin and thus appears to play a role in mitogenic signaling events, especially in the mechanism of v-Src oncogenesis (Sargiacomo et al., 1994).

The observation of a relationship between elevated CK2 expression and hence increased activity as well as the proliferative state of the tumor is so far a phenomenological observation. It has been shown that overexpression of CK2α leads to an increase in the frequency of lymphomas, although only to a modest extent, but it is significantly increased by coexpression with c-Myc (Landesman-Bollag et al., 1998). A similar mechanism is postulated by Kélliher et al., (1996) for Tal-1 action in Tal-1/CK2α thymocytes. Expression of the Tal-1 transgene leads to lymphoid malignancy in mice that is accelerated by the presence of the CK2α transgene. The Tal-1 gene product is a serine phosphoprotein and bHLH (basic helix-loop-helix) transcription factor known to regulate embryonic hematogenesis (Kelliher et al., 1996). It has been suggested that there are two transcriptional regulatory systems that collaborate with CK2 dysregulation

and may serve as candidates to human lymphoid malignancies and pathogenicity (Guerra and Issinger, 1999). The first involves the bHLH transcription factor Myc. CK2 phosphorylates both Myc and its DNA binding partner Max (Bousset et al., 1993). Phosphorylation by CK2 inhibits DNA binding by Max/Max homodimers but does not affect DNA binding by Myc/Max heterodimers, thus favoring gene transcription (Berberich and Cole, 1992). The second candidate system comprises the TAL/LMO heterodimeric transcriptional regulators. LMO-2 is a zinc finger transcription factor with an essential function in erythropoiesis (Hwang and Baer, 1995). Tal-1 heterodimerizes with LMO proteins to form heterodimeric complexes that regulate transcription in early erythroid and myeloid cells and are essential for their development (Warren et al 1994). The Tal-1 and LMO-1 are two genes that are frequently rearranged or ectopically expressed in human T- cell acute lymphoblastic leukemia (Hwang and Baer, 1995). CK2 phosphorylates Tal-1 and overexpression of CK2α collaborates with Tal-1-induced T-cell acute leukemia (Kelliher et al., 1996).

The tumor suppressor p53 can be regulated by CK2. Landesman-Bollag et al. (1998) have reported that the deficiency of the tumor suppressor p53 and misexpression of protein kinase CK2α collaborate in the development of thymic lymphoma in mice. In addition, it has been suggested that the dysregulation of CK2 might collaborate with the loss of a p53 allele to affect lymphocyte development or transformation (Xin et al., 1999). p53 is a target of CK2 phosphorylation, it has been shown that CK2 interacts and phosphorylates human p53 at Ser-392 (Ser-386 in murine p53) (Meek et al., 1990; Herrmann et al., 1991). The sequence surrounding this phosphorylation site is

XXXD$^{392/386}$SD (NCBI, Genbank accession # BC003596). The interaction between p53 and CK2 is discussed in more detail below.

1.2. p38 Mitogen-Activated Protein kinase (p38 MAP kinase)

1.2.1. Overview

The mammalian p38 MAP kinase family consists of at least four different homologous proteins, p38α, p38β, p38γ and p38δ (Widmann et al., 1999). The different isoforms display distinct expression patterns (Tibbles and Woodgett, 1999; Wang et al., 1997) and data indicating isoform-specific activities have been reported (Wang et al., 1998). However, the precise biochemical function that each isoform serves *in vivo* remains unclear.

The p38α and p38β genes are ubiquitously expressed. However, p38γ and δ are differentially expressed in different tissues. p38γ is predominantly expressed in skeletal muscle and p38δ is enriched in lung, kidney, testis, pancreas and small intestine (Wang et al., 1997). p38γ expression was reported to be induced during muscle differentiation and p38δ expression was shown to be developmentally regulated (Ono and Han, 2000).

p38α MAP kinase is activated by diverse cellular stress conditions (microtubule inhibitors, ultraviolet and γ irradiation, osmotic shock, heat shock, oxidative stress, lipopolysaccharide, protein synthesis inhibitors) (Takenaka et al., 1998; Tibbles and Woodgett, 1999), certain cytokines (interleukin-1, -2, -3, -7, -8; [IL-1, IL-2, IL-3, IL-7, IL8], tumor necrosis factor; TNFα), growth factors like granulocyte macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF), nerve growth factor

(NGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and G protein-coupled receptors (Widmann, 1999; Ono and Han, 2000; Mainiero et al., 2000). Table 2 lists some stimuli reported in recent years that lead to the activation of p38α.

Although the other three p38 group members, p38β, p38γ and p38δ were cloned several years ago, there is little information concerning their activation in different cells under diverse conditions (Ono and Han, 2000).

1.2.2. Regulation

p38 MAP kinase is activated by dual phosphorylation of threonine and tyrosine residues in the Thr-Gly-Tyr activation motif between kinase subdomains VII and VIII (Widmann et al., 1999). Stimulation of p38 involves the activation of several dual protein kinases, the MAP kinase kinases (MKKs), including MKK3, MKK4 and MKK6, which have been reported to phosphorylate p38 *in vitro*. There are reports indicating that distinct upstream kinases selectively activate p38 isoforms. MKK3 cannot effectively activate p38β like MKK6 does; p38δ can be activated by MKK3 and MKK6, and also by the c-Jun N-terminal kinase (JNK) kinases, MKK4 and MKK7 (Derijard et al., 1995; Han et al., 1996). These data indicate that the activation of p38 isoforms can be both specifically regulated through different regulators and coactivated by the same upstream regulators.

p38 MAP kinase can phosphorylate and activate the MAP kinase-activated protein (MAPKAP) kinase, 2 and 3, which in turn phosphorylate small heat-shock proteins such

as the 27-kDa heat shock protein (HSP27) (Paul et al., 1997). Other substrates for p38 have been identified including HSP70 and transcription factors such as, ATF2, Elk, CHOP (also known as GADD 153), MEF2C and CREB (Hung et al., 1998; Widmann et al., 1999). Because p38 MAP kinase can phosphorylate many different substrates, it is reasonable to suggest that p38 affects many different biological functions. To investigate the biological function of p38 MAP kinase, a kinase inhibitor of p38 [pyridinyl-imidazole compound SmithKline Beecham (SB) 203580] is often used. It has been shown that SB 203580 is extremely specific for p38α and p38β isoforms *in vitro* and *in vivo* (Cuenda et al., 1995; Beyart et al., 1996; Cuenda et al., 1997; Engelman et al., 1998) and has no inhibitory activity on all kinases tested including *in vitro*, including the other MAP kinases (Engelman et al., 1998). The crystal structure of p38 in complex with SB 203580 revealed that the inhibitor binds within the ATP pocket of the kinase and this binding interferes with the p38-substrate interaction (Tong et al., 1997; Young et al., 1997). It has been shown that a single amino acid substitution within the ATP binding pocket of p38 MAP kinase alters the sensitivity of p38 to the inhibitors and offers an explanation for the specificity of this class of inhibitors (Gum et al., 1998).

It was found that the administration of SB 203580 blocks the production of cytokines (IL-1 and TNFα) in stimulated monocytes (Lee et al., 1994). On the other hand, inhibition of p38 MAP kinase by SB 203580 prevents IL-2 and IL-7 –driven T-cell proliferation, indicating a role for p38 in cytokine-stimulated cellular proliferation (Crawley et al., 1997). On the other hand, p38 MAP kinase has been shown to be implicated in apoptosis (Widmann et al., 1999). Several cellular stresses such as osmotic shock and ultraviolet irradiation that cause apoptosis, also activate p38 MAP kinase

activity (Whitmarsh et al., 1996). Blockage of p38 MAP kinase activity by SB 203580 impaired p38-mediated apoptosis in T cells (Ichijo et al., 1997).

Recently, p38 MAP kinase, but not Erk1, Erk2 or JNK, was shown to be activated in mammalian cultured cells when the cells were arrested in M phase by disruption of the spindle with nocodazole (Takenaka et al., 1998). Addition of activated recombinant p38 to Xenopus cell-free extracts caused arrest of the extracts in M phase, and injection of activated p38 into cleaving embryos induced mitotic arrest. Treatment of NIH 3T3 cells with a specific inhibitor of p38 suppressed activation of the checkpoint by nocodazole. Thus, p38 functions as a component of the spindle assembly checkpoint in somatic cell cycles (Takenaka et al., 1998). In addition, p38 has been shown to interact with the tumor suppressor p53 and phosphorylates the murine C-terminal region of murine p53 at Ser-389 (Ser-392 in human) and the N-terminal region at Ser-33 in response to genotoxic stress such as UV and γ-radiation (Bulavin et al., 1999; Keller et al., 1999; Sanchez-Prieto et al., 2000). This phosphorylation has been shown to affect p53-DNA binding and transcriptional activity (Keller et al., 1999; Sanchez-Prieto et al., 2000).

How p38 MAP kinase participates in so many different biological processes remains unknown. It has been suggested that the multiple functions and different responses of p38 may be due to the availability of downstream substrates (Paul et al., 1997). Nebreda and Porras (2000) have stated that the critical determinant of understanding the molecular mechanisms that allow p38 MAP kinase to produce different cellular behaviors, is the identification of the physiologically relevant p38 target or targets.

Table 2. Extracellular stimuli of p38 and functional outcome (Ono and Han 2000).

Stimuli	Functional outcome
Pathogens	
LPS	
Soluble staphylococcal peptidoglycan (pPGN)	
Plasma-opsonized *Staphylococcus aureus* (S-SA)	H_2O_2 production
Superantigen staphylococcal enterotoxin B (SEB)	TNF-α production
Macrophage-activating lipopeptide (MALP)-2	IL-1, TNF production
Mycoplasma fermentas lipid-associated membrane	IL-8 production
Echovirusl (EVI)	Jun B expression
Murine hepatitis virus strain 3 (MHV-3)	Macrophage
prothrombinase	production
Simian immunodeficiency virus	
Herpes simplex virus lype-I (HSV-1)	
Clostridium botulinum C, toxin	c-Jun expression
Cytokines	
TNF-α	
IL-1	
IL-2. IL-7	Proliferation
IL-17	Cyclooxygenase
(COX)-2	And IL-8 production
IL-18	HIV type I and IL-8 production
TNF-α and GM-CSF	Respiratory burst response
Growth factors	
TGFβ	Chemotaxis, actin organization

Table 2. Continued

Stimuli	Functional outcome
NGF	Differentiation
NGF withdrawal	Apoptosis
PDGF	Proliferation
VEGF	Actin organization,
Stress environment	
Heat shock	
Hyperosmolarity	
Hypoosmolarity	
Ultraviolet	
Oxygen radical metabolites (H_2O_2, menadione)	
Stretch cardiac myocyte	Cardiac hypertrophy
Cyclic stretch	Proliferation
Hypoxia	
Ischemia/reperfusion	Heart Repair
As, V, Zn and Cd	
Arsenite	ERK activation
Others	
CD40 cross-linking	NF-κB activation, B cell proliferation
B cell antigen receptor cross-linking	
Okadaic acid	Collagenase-I
(MMP-1)	expression
Phenylephrine	
Norepinephrine	Differentiation
Carbachol	HSP phosphorylation
Angiotensin 11	Vascular smooth
muscle cell	Proliferation, Na^+/H^+ exchange

Table 2. Continued

Stimuli	Functional outcome
PAF	
Synthetic oligonucleotides (CpG DNA)	
Adenosine	Ischemic heart Preconditioning
Ceramide (C2, C6 and SMase)	COX-2 production
S-nitrosoglutathione	
CD473 (retinoid)	
Calphostin-C	
Anisomycin	
Sodium salicylate	Apoptosis
Taxol	
Glutamate	Apoptosis
Gonadotropin-releasing hormone (GnRH)	
Cholecystokinin (CCK)	Actin organization
GM-CSF	Development of hemopoietic cells
Steel locus factor (SLF)	Development of hemopoietic cells
FGF	Regulation of gene expression
IGF	Anti-apoptosis, gene Expression
Endothelin-I	Cardiac myocyte Hypertrophy
Thrombin	
Three dimensional collagen	Collagenase-3 (MMP-13) expression

1.3. The Tumor Suppressor p53

1.3.1. Overview

A large body of evidence places the transcription factor p53 at a critical point of converging pathways from diverse cellular insults, which can elicit coordinated cellular responses that result in adaptation to these insults (Hall et al., 1996a). Much important information have been derived from the analysis of clinical biopsy material, and this indicates that abnormalities of p53 are among the most prevalent molecular abnormalities in human (and other animal) cancer, with missense point mutations in the sequence-specific DNA binding domain of p53 and allelic loss at the p53 locus on chromosome 17 being particularly prevalent. In addition, p53 is a frequent target for inactivation by virally encoded proteins such as SV40 large T antigen, the HPV E6 ORF, hepatitis B X antigen, and adenovirus E1a (Hall et al., 1996a; Prives and Hall, 1999).

A wealth of information has been well documented concerning p53 and its role in normal cells in maintaining the integrity of the genome and in neoplasia (Ko and Prives, 1996; Prives and Hall, 1999). p53 suppresses tumor growth by two distinct mechanisms. First, p53 induces apoptosis, a tightly regulated pathway of programmed cell death involving changes in mitochondrial physiology, cysteine protease activation and DNA degradation (Miyashita and Reed, 1995; Moll and Zaik, 2001; Shen and White, 2001). p53 also prevents growth by halting the cell cycle (Prives and Hall, 1999). p53 inhibits G1/S transition in cells exposed to DNA damage by causing accumulation of p21WAF1 (El-Deiry et al., 1995). p53 also regulates a mitotic spindle checkpoint that prevents DNA synthesis before chromosome segregation (Cross et al., 1995). In addition, p53

controls a G2 checkpoint and expression of the wild-type protein can prevent G2/M transition in mouse and human cell lines (Prives and Hall, 1999; Taylor and Stark, 2001). Loss of these checkpoints in p53 deficient cells can lead to polyploidy, chromosomal breaks and centrosome amplification, indicating that regulation of p53 contributes to the maintenance of DNA integrity (Cross et al., 1995; Fukasawa et al., 1996).

The biology of p53 and its role to maintain the genomic stability of the cell through both cell cycle arrest and apoptosis is discussed below.

1.3.2. p53 structure and function

The transcription factor p53 is a modular protein with several regions with distinct, but interdependent, functions (Arrowsmith and Morine 1996; Soussi and May, 1996). These functions are coordinated such that the p53 protein within a cell acts to integrate signals emanating from a wide range of cellular stresses, and allows the cell to respond to these insults by activating a set of genes whose products facilitate adaptive and protective activities, including apoptosis and growth arrest (Arrowsmith and Morine, 1996; Ko and Prives, 1996; Soussi, 1996). To function in such a complex manner, p53 protein is subject to a diverse array of regulatory mechanisms that keep this tumor suppressor protein in check until needed (Ko and Prives, 1996; Prives and Hall, 1999; Taylor and Stark, 2001). The biochemical properties that underpin the complex biology are intimately correlated with the structure of the p53 molecule.

The core of the p53 protein is a region (within residues 100-300) which folds in such a way so as to form a domain which can interact with DNA in a sequence-specific

manner (El-Deiry et al., 1992; Bourdon et al., 1997; El-Deiry et al., 1998a). The majority of missense mutations seen in human (and animal) tumor occur in regions of the gene encoding this domain such that either critical residues involved in DNA contact are altered or that the whole conformation of the core domain of the protein is disrupted. The consequence of either of these events is the loss of the ability of p53 to specifically bind to DNA in a sequence-specific manner. The binding to DNA is optimal when the protein is in a tetrameric state as a consequence of interactions of four separate p53 molecules via the tetramerization domain (Jeffrey et al., 1995). The C terminal region is composed of predominantly basic residues and forms a region that has key regulatory properties. This region undergoes modification by acetylation, phosphorylation, O-glycosylation, and RNA binding has been also reported, but the physiological significance of these posttranslational modifications remains uncertain (Prives and Hall, 1999). The DNA binding domain is separated from the transcriptional activation domain by a region containing a series of repeated proline residues typical of a polypeptide that can interact with signal transduction molecules that contain a SH3 (src-homology domain-3) binding domain (Goria et al., 1996). Through this domain p53 is influenced by diverse signaling molecules including the c-abl oncogene (Yuan et al., 1996). The acidic N-terminal transcriptional activation domain allows p53 protein, in the context of its specific binding to a target DNA sequence, to recruit the basal transcriptional machinery required for transcribing new mRNA and by so doing, activate the expression of target genes. This region is also critically involved in regulating the stability and activity of p53 protein via interactions with proteins such as Mdm2 (murine double minute clone 2), which allows targeting of p53 to the ubiquitin-mediated proteolytic machinery (Ko and Prives, 1996).

Mdm2 binding also blocks the ability of p53 to interact with the transcriptional apparatus (Prives and Hall, 1999). Modification of p53 by phosphorylation may alter many of these properties and, in particular, the interaction of p53 with other proteins such as Mdm2. By this means, much of the regulation of the p53 pathway occurs.

1.3.3. Regulation

p53 is a nuclear phosphoprotein, and several protein kinases have been demonstrated to be able to phosphorylate p53 at sites believed to be of physiological relevance. However, it is not clear how cellular stresses communicate and regulate p53 function. There are a number of phosphorylation sites on p53 that are altered after DNA damage and, in some cases, new protein kinases have been shown to be able to phosphorylate p53 at these sites. Furthermore, such phosphorylation events have been shown to result in alterations in the protein which make it more stable and more active. Recently, it has been shown that DNA-damage induced phosphorylation of p53 may achieve optimal p53 activation and stability by inhibiting MDM2 binding to p53 protein (Zhang and Xiong, 2001). Phosphorylation, however, is not the only way in which p53 is regulated. Interestingly, another form of p53 modification, acetylation, has also been demonstrated and shown to be DNA damage-inducible (Gu et al., 1997) and the two may be temporally and mechanistically linked (Hupp, 1999).

1.3.3.1. N-terminal phosphorylation

The N-terminus region of p53 consists of two transcription activation subdomains extending between residues 1 and 63, and a region spanning residues 60–97, containing several copies of the sequence PXXP, which plays an important role in the induction of apoptosis by p53 (Ko and Prives, 1996). Several phosphorylation sites have been mapped within the N-terminus of human and/or murine p53 including, casein kinase I (CK I) sites on murine p53: Ser-4, Ser-6, and Ser-9; and DNA-activated protein kinase (DNA-PK) sites: murine and human Ser-15 and human Ser-37 (Meek, 1994; Jayaraman and Prives, 1999). Ser-33 in human p53 is phosphorylated *in vivo,* and at least one protein kinase, the TFIIH-associated trimeric cyclin-activating kinase complex (CAK), consisting of cyclin H, CDK7, and p36/MAT1, has been shown to phosphorylate p53 at that site *in vitro* (Prives and Hall, 1999). By contrast, murine p53 is phosphorylated at residues 33 and 34 *in vivo* and can be phosphorylated *in vitro* by p38 MAP kinase JNK, respectively (Keller et al., 1999; Milne et al., 1995). JNK can also phosphorylate human p53 at other N-terminal sites, although the exact site is unknown (Adler et al., 1997). In addition, the amino terminal region of p53 can be phosphorylated *in vitro* by Erk1 and Erk2 at residues Ser-73 and Ser-83 (Adler et al., 1997). It should be noted that there are a number of other potential threonine and serine phosphate acceptors within the N-terminal region of p53. Although two-dimensional phosphotryptic mapping has provided strong support for the identification of the known sites, and perhaps can be tentatively used to rule out others, it remains possible that in some cells or under some conditions, novel sites may be utilized and such sites may indeed turn out to be very interesting. Although

demonstration of the existence of sites and relevant kinases is important, showing that a given site is phosphorylated *in vivo* in response to a given stimulus takes us considerably further in understanding the biological relevance of such modifications. A barrier to determining stress-induced changes in phosphorylation is that it is difficult to compare accurately the phosphorylation status of p53 from normal cells, where it is often present in nearly undetectable amounts, with that in stressed cells, where its levels have risen dramatically, often by one or two orders of magnitude (Prives and Hall, 1999). One important technical advance has been the use of phosphorylation site-specific antibodies. These reagents have made possible the rapid identification of specific phosphorylation events, either constitutive or inducible, and a number of groups have now reported advances with such antibodies. The first site to be shown to be inducibly phosphorylated after DNA damage was Ser-15 (Siliciano et al., 1997), as mentioned above, previously demonstrated to be a substrate for the DNA-dependent protein kinase (DNA-PK) (Lees-Miller et al., 1992). Recently, another kinase, the ATM (ataxia telangiectasia mutated) protein, the product of A-T gene (the human genetic disorder ataxia telangiectasia) has also been shown to be able to phosphorylate this site (Banin et al., 1998; Canman et al., 1998) and it is likely that additional kinases will prove capable of carrying out this modification as well. Importantly the activity of the ATM protein kinase is significantly increased when cells are subjected to DNA damage. However, the observations that several protein kinases can phosphorylate a DNA damage-responsive site leave critical questions to be solved. It is still unknown which is the primary kinase that actually phosphorylates p53 at this (or any) site *in vivo*. To make matters even more complicated, it is now known that Ser-15 is not the only DNA damage-inducible site on p53. It was

recently shown that both Ser-33 and Ser-37 are also DNA damage-inducible (Jayaraman and Prives, 1999; Keller et al., 1999). Furthermore, the Meek laboratory has identified additional protein kinases for which p53 is a substrate whose activities are DNA damage-inducible (Milne et al., 1995). Moreover, Silicano et al. (1997) reported, in addition to Ser-15, another site within the first 20 amino acids of p53 that was increased in irradiated cells.

What is the significance of phosphorylation of N-terminal sites? First, it is often the case that modification of a given site leads to conformational changes that are propagated to other regions of the phosphorylated protein. Second, p53 makes specific contact with a number of proteins (see below) and with DNA, and phosphorylation can affect these interactions. The N-terminus of p53 associates with both components of the transcriptional machinery, with TAFs (TATA box binding protein associated factor), and with its negative regulator, Mdm2 (Jayaraman and Prives, 1999; Prives and Hall, 1999). Shieh et al. (1997) demonstrated that phosphorylation of p53 with DNA-PK *in vitro* both markedly reduces the ability of p53 to bind to Mdm2 and causes a significant change in conformation of the N-terminus. Mdm2 both destabilizes p53 and represses transactivation by p53, and so this result provides a plausible explanation for how p53 is induced after DNA damage. It is likely that additional N-terminal DNA damage-inducible modifications will also be shown to affect the interactions of p53 with Mdm2, and it was recently reported that phosphorylation of human p53 by JNK inhibits its interaction with Mdm2 (Fuchs et al., 1998). It is also possible that phosphorylation at N-terminal sites will be shown to affect its interaction with transcription factors, as it was

reported that phosphorylation at Ser-15 and Ser-37 decreases the ability of p53 to bind to TFIID (Jayaraman and Prives, 1999).

1.3.3.2. C-terminal phosphorylation

The C-terminus of p53 has been described both as a region whose function is to control sequence-specific DNA binding and as a DNA damage recognition domain (Ko and Prives, 1996). It possesses autonomous DNA binding and strand reassociation abilities but to date, there has been no evidence of sequence specificity in these interactions (Ko and Prives, 1996). The p53 protein exists in solution predominantly as a tetramer and tetramers are the most efficient DNA binding form of the protein (Hupp, 1999). The structure of the oligomerization domain of human p53 reveals a β-sheet-turn–α-helix motif which forms a pair of dimers (Hupp, 1999). Mutation of the p53 tetramerization region substituting residues with alanine or hydrophobic amino acids leads to altered oligomerization properties (Hupp, 1999). Substitution of this region with a heterologous tetramerization domain, however, produces a protein with many of the activities of wild-type p53 (Hupp, 1999; Jayaraman and Prives, 1999). It is interesting that disruption or loss of oligomerization function is associated with loss of cell cycle arrest but not growth suppression as measured by colony formation assays (Ishioka et al., 1995; Moll and Pellegata, 1995). Recently mutations have been identified in this domain (Lomax et al., 1997).

The effects of covalent modification of the C-terminal region of p53 on its DNA binding properties continue to spark much interest. Hupp et al. (1992) were the first to

discover that phosphorylation, antibody interaction or deletion of the C-terminus stimulates DNA binding of inert bacterially expressed p53, and this pivotal observation has led to a plethora of related findings. It is now known that phosphorylation at CK2 sites (Ser-392) or PKC (protein kinase C) sites (Ser- 371, 376, and 378) within this region result in stimulation of binding (Ko and Prives, 1996). The mechanism by which the C-terminus regulates DNA binding is not yet fully understood. Two models have been proposed: in the first, it has been hypothesized that the C-terminus allosterically regulates conversion of p53 from an inert 'latent' form to one which is active for DNA binding (Hupp et al., 1994). Evidence for this model is based on the observation that p53 can be isolated from baculovirus-infected insect cells or synthesized in vitro in a form that binds DNA only weakly but which, upon treatments as outlined above, becomes activated. The second model postulates that binding by the central domain is hindered by the interactions of the C-terminus with longer DNA molecules (Bayle et al., 1995; Anderson et al., 1997). It is not clear at this point which of these two models (or both) is correct. Validation of the allosteric model will require physical evidence for a conformational alteration in p53 protein upon one or more of the modifications noted previously. Indeed, it is entirely possible that both explanations contribute to the way in which p53 is regulated by its C-terminus.

There have been a number of current developments in understanding how phosphorylation regulates p53 DNA binding. We must preface this optimistic statement by noting that attempts to discern phenotypes of phosphorylation site mutants in p53 have been confusing at best and disappointing at worst. Mutation of murine phosphorylation sites at N- and C-termini have frequently yielded p53 proteins which are roughly

equivalent to wild-type protein in standard transient transfection assays of transcriptional activation of p53- responsive reporters (Ko and Prives, 1996). Hao et al. (1996), however, reported that wild-type p53 in G1-arrested murine cells is inactive, while mutation of the murine CK2 site to a charged residue (Glu) rescued p53 function. Additionally, mutation of the murine CK2 site to alanine had little effect on transactivation of the cyclin-dependent kinase inhibitor, p21, and RGC (ribosomal gene cluster) reporters, while it markedly inhibited the ability of p53 to repress the SV40 promoter (Hall et al., 1996a). Although it is not yet well understood, we might speculate that an interaction of the C-terminus with a protein component of the general transcription machinery such as TBP or p300, both of which have been implicated in the repression function of p53, either might require p53 to be phosphorylated at that site or might be affected by mutating Ser-392 (Prives and Hall, 1999). Further insight into the mechanism by which p53 is stimulated by CK2 is derived from the observation that p53 is bound stably by the beta subunit of CK2 (Appel et al., 1995; Hall et al., 1996b) and after phosphorylation, p53 can no longer reanneal DNA (Filhol et al., 1996). This interaction, as well as phosphorylation, may contribute to the effect of the protein kinase on p53 DNA binding. Inhibition of p53 annealing of DNA by CK2 phosphorylation supports the proposal that the C-terminus, when bound to DNA, interferes with specific binding by the central region. Note, however, that Sakaguchi et al. (1998) have demonstrated that peptides spanning the C-terminal protein (residues 303–393) display a ten-fold greater association constant for reversible tetramer formation after phosphorylation by CK2. Whether this striking observation is also true for full length p53 awaits validation by experimentation. Importantly, Kapoor and Lozano (1998) and

Lu et al. (1998), using antibodies specific for p53 phosphorylated at the CK2 site, showed that UV irradiation, but not gamma irradiation (IR), induces phosphorylation at this site.

Another binding site at the C-terminal of human p53 is for CDK. It has been identified within residues 330– 339 (Wagner et al., 1998). Interestingly, substitution of Ser-392, but not Ser-315, with the acidic residue Asp led to reduced interaction of CDK with p53 (Sakaguchi et al., 1998). Reciprocally, whereas phosphorylation of Ser-392 increases tetramerization, this effect is reversed when p53 is phosphorylated at Ser-315 (Sakaguchi et al., 1998). Taken together, these data indicate an inter-dependent relationship between the CK2 and CDK sites on p53.

1.3.3.2. Responses to the activation of p53: Downstream events

The downstream events mediated by p53 culminate in either cell cycle arrest or maintenance of chromosomal diploidy or mitotic fidelity when cells are exposed to genotoxic stresses.

I will discuss briefly those events that pertain to the objective of this thesis.

1.3.3.2.1. p53 and cell cycle checkpoints

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the transformation of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in response to DNA damage, replication blocks, and spindle damage. Checkpoint pathways have components shared among all eukaryotes, underscoring the conservation of cell cycle regulatory machinery. Checkpoints are regulated by several cell cycle transitions that are dependent upon the activity of cyclin-dependent kinases (Cdks), and inhibition of these kinases is a common mechanism by which cell cycle arrest. These enzymes are composed of a kinase subunit, Cdk, and an activating subunit, cyclin, in a complex that is subject to many levels of regulation (Villard et al., 2001).

Although, the most well-understood component of p53 function is its ability to induce a G1 arrest as a result of DNA damage, several recent reports have implicated a role for p53 in G2/M and the spindle assembly checkpoints (Taylor and Stark, 2001).

In response to DNA damage, the DNA-damage checkpoint detects damaged DNA and generates a signal that arrests cells in the G1 (Gap1) phase of the cell cycle, slows down S phase (DNA synthesis), arrests cells in the G2/M (Gap2/Mitosis) phase, and induces the transcription of repair genes. The most well-understood component of DNA damage checkpoint is p53 function for its ability to induce a G1 arrest via p21 (WAF1) pathway. In response to DNA damage, p53 has been shown to induce the expression of the p21(WAF1) CDK inhibitor, which appeared to be directly controlled by p53 transcription activation through two specific p53 DNA-binding response elements located within the p21 promotor (El-Deiry, 1995; El-Deiry et al., 1998b; Liu and Kulesz-Martin, 2001). Recent studies have revealed the possible existence of distinct signaling pathways that may preferentially utilize one or more of the other p53-binding elements within the p21 promotor (Resnick-Silveman, 1998). Once activated, p21 protein binds to cyclin-CDK complexes and inhibits their kinase activity (El-Deiry et al., 1998a; Liu and Kulesz-Martin, 2001). Because CDK kinase activity is required for various cell cycle transitions, p21 is a potent cell cycle inhibitor. p21 binds with its greatest affinity to G1 cyclin-CDK complexes, and binds poorly to cyclin B/cdc2 during mitosis (El-Deiry et al., 1998a). p21 is a member of the p27 and p57 KIP/CIP family of universal cell cycle inhibitors which can inhibit cyclin D/CDK4/6 complexes during G1/S and G2 cell cycle transition (Pines, 1999). p21 also binds to the proliferating cell nuclear antigen (PCNA) and can inhibit the processivity of DNA replication (El-Deiry et al., 1998b).

In mitosis, the proper segregation of chromosomes requires the execution of a number of processes: a bipolar spindle must be assembled; chromosomes must attach to the spindles through the kinetochore, a protein structure that forms on the centromeres of chromosomes. The kinetochores of sister chromatids must bind to spindle fibers attached to opposite poles and the properly attached chromosomes must arrive at the metaphase plate. The spindle assembly checkpoint prevents the onset of anaphase, the actual segregation of chromosomes, until these processes have been properly accomplished. Once these events take place, cells can execute anaphase and daughter cells separate (cytokinesis) and then progress into the next cell cycle (Pines, 1999; Gardner and Burke, 2000; Villard et al., 2001).

Disruption of the spindle with microtubule-depolymerizing drugs such as nocodazole and colchicine arrest cells in mitosis (Runder and Murray, 1996), and this arrest depends on the integrity of the spindle assembly checkpoint. The induction of mitotic arrest by the effect of the microtubule-depolymerizing drugs has been shown to be related to the elevated level of activation of the cyclin-dependent kinase-1 (CDK-1); cdc2/cyclin B complex. Adaptation to prolonged checkpoint arrest can occur by inhibitory phosphorylation of cdc2 or degradation of cyclin B (this effect is discussed below).

The cell cycle G2/M transition and progression through mitosis is driven by the kinase activity of a complex referred to as maturation- or M-phase-promoting factor (MPF) or CDK-1. This complex consists of a catalytic subunit (34-kDa cyclin-dependent kinase, cdc2), a regulatory subunit (cyclin B proteins), and associated proteins (Norbury and Nurse, 1992). Entry into mitosis requires cdc2 activation, a process that depends

upon an increase in cyclin B expression and the dephosphorylation of cdc2. Progression through mitosis and cytokinesis requires the subsequent inactivation of cdc2, which depends in part on cyclin B degradation. Experiments with yeast indicate that the mitotic spindle cell cycle checkpoint feeds into the cell cycle regulatory machinery at mitosis by a pathway that delays the degradation of cyclin B and maintains cdc2 kinase activity (Elledge, 1996; Villard et al., 2001). Thus, cyclin B is degraded and cdc2 is inactivated only after certain aspects of mitosis related to spindle assembly and disassembly are properly completed.

Human CDK-1 was identified through complementation of a cdc2 mutant in yeast and as a protein that cross-reacted with antibodies to yeast cdc2 (Mowal and Stewart, 1998). Cyclin B has been described as a protein with no enzymatic activity and whose level increases during the cell cycle and then is destroyed late in mitosis; it is essential for cdc2 kinase activation and specificity (Norbury and Nurse, 1992; Ewen, 2000).

Cdc2 activation is a multi-step process that begins when it binds to its regulatory subunit cyclin B, whose levels rise during G2 and peak in mitosis. Activation of cdc2 requires both cyclin binding and phosphorylation at position Thr-161 by cyclin-activating kinase (CAK) (Norbury and Nurse, 1992; Mowal and Stewart, 1998). CAK kinase was first found in *Xenopus* pM015 kinase and has sequence similarity to cdc2, now called CDK7 (Solomon et al., 1993; Mowal and Stewart, 1998).

The phosphorylation of cdc2 at Thr-161 has been suggested to enhance binding of cyclin B (Mowal and Stewart, 1998). This is based on the structural analysis of the CDK2/cyclin A complex which shows that in the absence of binding to cyclin A, the regulatory T loop that is located at the ATP-binding site, blocks protein substrate binding

(Russo et al., 1996). Cyclin binding reorientates the ATP-binding site and allows movement of the T-loop to allow substrate binding and make it more accessible for phosphorylation by CAK (Russo et al., 1996). Phosphorylation of Thr-160 (equivalent to Thr-161 in cdc2) in the T-loop moves the loop further and stabilizes CDK2/cyclin A by increasing the number of contacts (Russo et al., 1996; Bartek and Lukas, 2001).

CDK1 kinase phosphorylates critical proteins involved in S phase and M phase transitions resulting in DNA synthesis, chromosome condensation, cytoskeletal reorganization, nuclear lamin disassembly, and cell shape changes (Norbury and Nurse, 1992; Ekholm and Reed, 2000). Some targets include histone H1, nuclear lamins, vimentin, a microtubule-associated protein 115, p53 protein and others (Mowal and Stewart, 1998).

Before mitosis, cyclin-B–cdc2 complexes are held in an inactive state by phosphorylation of cdc2 at Thr-14 and Tyr-15, which is catalyzed by the protein kinases Wee1 (which phosphorylates Tyr-15 only) and Myt1 (which phosphorylates both Thr-14 and Tyr-15) (Pines, 1999; Ewen, 2000; Smits and Medema, 2001). Dephosphorylation is carried out by the dual-specificity protein phosphatase Cdc25C. Cdc2 activation at the onset of mitosis results from the concurrent inhibition of Wee1 and Myt1 and stimulation of the phosphatase Cdc25C (Pines, 1999; Ewen, 2000; Smits and Medema, 2001). Activation of Cdc25C requires phosphorylation of several sites in the Cdc25C amino-terminal domain; this is catalyzed by at least two kinases. One of these kinases appears to be the Polo-related kinase (Plk); the *Xenopus* Plk homolog, Plx, phosphorylates and activates Cdc25C *in vitro* and is required for Cdc25C activation in frog egg extracts (Kumagai and Dunphy, 1996). Activating phosphorylation of Cdc25C is also carried out

by the Cyclin-B1–cdc2 complex itself (Hoffman et al., 1993; Smits and Medema, 2001). The ability of cdc2 to activate its own activator provides the potential for a positive feedback loop in Cdk1 activation, whereby partial activation of Cdc25C (perhaps by Plk) could lead to complete and overwhelming cdc2 activation. This positive feedback loop may be enhanced by the ability of cdc2 to phosphorylate and inactivate Wee1 (Patra et al., 1999). There is also evidence that cdc2 stimulates Plk activity in frog egg extracts, providing another potential mechanism for positive feedback (Abrieu et al., 1998).

There are two different cyclin B proteins in mammalian cells. Cyclin B2 is a non-essential protein that associates with the Golgi apparatus and probably plays a role in Golgi remodelling during mitosis (Jackman et al., 1995; Brande et al., 1998). Cyclin B1 is an essential protein that is thought to be responsible for most of the other actions of cdc2 in the cytoplasm and nucleus (Brande et al., 1998; Villard et al., 2001).

Recent data have indicated a role for the p53 tumor suppressor in regulating G2 and the mitotic spindle cell cycle checkpoints to maintain DNA integrity. It has been shown that p53 may interfere with the control of cyclin B metabolism by the mitotic checkpoint pathway and facilitate the accumulation of cells with a polyploid DNA content (Hixon et al., 1998). In addition, studies by Taylor et al. (1999) have shown that overexpression of p53 causes G2 arrest, attributable in part to the loss of cdc2 activity. Transcription of cdc2 and cyclin B1, determined using reporter constructs driven by the two promoters, was suppressed in response to the induction of p53. Suppression requires the regions -287 to -123 of the cyclin B1 promoter and -104 to -74 of the cdc2 promoter. p53 did not affect the inhibitory phosphorylations of cdc2 at Thr-14 or Tyr-15 or the activity of the cyclin-dependent kinase that activates cdc2 by phosphorylating it at Thr-

161. Overexpression of p53 was shown also to interfere with the accumulation of cdc2/cyclin B1 in the nucleus, required for cells to enter mitosis (Taylor et al., 1999). Along a similar theme, Park et al. (1999), have shown that over expression of p53 in human bladder carcinoma cells released from G1/S arrest, causes them cells to become arrested at G2/M transition due to repression of cyclin B1 level. It has been shown that p53 can inhibit cyclin B1 promotor activity and it is likely that the p53 mediates a decrease in cyclin B1 mRNA that result from a reduction in the rate of cyclin B1 transcription (Innocente et al., 1999).

Several laboratories have reported that the loss or mutation of p53 result in cellular polyploidy and loss of mitotic fidelity, implicating an important role of p53 in the mitotic spindle cell cycle checkpoint (Cross et al., 1995; Fukasawa et al., 1996).

It has been shown that fibroblasts isolated from individuals with Li-Fraumeni syndrome have a marked tendency to become heteroploid (or accumulation of more than one round of DNA without proper chromosomal segregation cell division, a process also called DNA endoreduplication) in culture (Bischoff et al., 1990). These individuals are born with heterozygous mutations in the p53 tumor suppressor gene (Livingstone et al., 1992). Heteroploidy is also commonly found in p53 null cells in culture (Harper et al., 1993) and *in vivo* (Cross et al., 1995) in p53 knockout mice. Intriguingly, overexpression of a mutant p53 protein on a p53 null background accelerated the appearance of polyploidy in a myelomonocytic cell line (Peled et al., 1996). Also, the expression of mutant p53 proteins in human colon carcinoma cells and murine cell lines causes karyotypic abnormalities, including an increase in ploidy levels during growth in culture (Agapova et al., 1996). It has also been shown that Li-Fraumeni syndrome fibroblasts

that carry heterozygous structural p53 mutant proteins progress to polyploidy when incubated in the presence of mitotic spindle inhibitors (Gualberto et al., 1998). However, normal human fibroblasts, p53 null Li-Fraumeni fibroblasts, and normal human fibroblasts infected with a retrovirus that expresses the human papillomavirus 16 E6, which binds to and promotes the degradation of p53, exhibit growth arrest when incubated in the presence of mitotic inhibitors (Gualberto et al., 1998). Although, Lanni and Jacks (1998) have recently reported that p53 null mouse fibroblasts have a normal mitotic spindle checkpoint, these fibroblasts may progress to polyploidy due to the inactivation of a p53-dependent postmitotic checkpoint (Lanni and Jacks, 1998). In addition, inactivation of wild-type p53 by the overexpression of a truncated (C-terminus) p53 protein in a murine prolymphocytic cell line leads to polyploidy (Minn et al., 1996).

1.3.3.2.2. p53 and centrosome homeostasis

Recently, it has been proposed that p53 actively participates in maintaining the stability of the genome through regulation of centrosome duplication or as a monitor that limits centrosome overproduction. Fukasawa et al. (1996), have reported that the regulation of the centrosome duplication cycle is abrogated in the absence of p53, resulting in hyperamplification of centrosomes.

In animal cells, the centrosome is composed of a pair of centrioles (core components of centrosome) and surrounding amorphous pericentriolar materials (Bornens, 1992). The centrosome, as a major microtubule organizing center, participates in various cellular events throughout the cell cycle. Functions of the centrosome are

perhaps most prominently featured during mitosis, where, two centrosomes become spindle poles and establish bipolarity, direct spindle microtubule assembly, and determine the cleavage furrow plane, all of which are required for the balanced segregation of chromosomes. In addition, during interphase the centrosome nucleates and spatially organizes the cytoplasmic microtubules, thus controlling redistribution of cellular organelles and establishment of cellular integrity (Bornens, 1992).

Because each daughter cell receives only one centrosome after mitotic decision, the centrosome must duplicate during the mitotic cycle of the daughter cell. In addition, the centrosome must duplicate only once before the subsequent cell division. The duplication cycle encompasses all phases of the cell cycle. Duplication begins near the G1/S boundary and is completed in G2 (Bornens, 1992). There are two major regulatory mechanisms imposed on centrosome duplication. One is the correct timing of initiation. The second is the prevention of reduplication of centrosomes once they have duplicated. Another important aspect of the centrosome duplication is that it always occurs in precise coordination with DNA replication. Loss of centrosome duplication cycle regulation or uncoupling of the centrosome duplication cycle from DNA replication cycle leads to abnormal amplification of centrosomes, which in turn profoundly affects mitotic fidelity (Bornens, 1992; Villard et al., 2001).

Control of both initiation of centrosome duplication and suppression of reduplication appears to be abrogated in p53 (-/-) mouse embryonic fibroblast cells (MEF) (Fukasawa et al., 1996). In these cells, Fukasawa et al. (1996) were able to show that centrosomes have undergone multiple cycles of duplication in a single cell cycle. In many cases, the abnormally amplified multiple copies of centrosomes co-migrated to the

poles, establishing pseudobipolarity. A small proportion of these cells with such pseudobipolar spindle underwent normal chromosome segregation and cytokinesis. However, a large proportion of cells with multiple spindle poles have been shown to undergo cell death in both *in vitro* culture of p53 (-/-) MEFs and *in vivo* p53 (-/-) mice (Fukasawa et al., 1996; Fukasawa, 1997; Tapore and Fukasawa, 2000).

1.3.3.2.3. p53 and regulation of apoptosis

Although the mechanisms of p53-dependent apoptosis are not clear, p53 plays a role in triggering apoptosis under several different physiological conditions. Normal thymocytes will undergo apoptosis in response to DNA damage, whereas thymocytes from p53 -/- mice do not undergo apoptosis in response to the same stimulus (Lowe et al., 1993b; Moll and Zaika, 2001). Similarly, in mice that are irradiated, the stem cells of the small and large intestines undergo apoptosis, which does not occur in p53-/- mice. However, it is equally clear that not all apoptotic events are p53-mediated. For example, immature thymocytes from p53 -/- mice die of apoptosis when exposed to glucocorticoids or compounds that trigger T cell receptor pathways (Lowe et al., 1993; Shen and White, 2001).

Physiological signals or activation of a specific signal transduction pathway can block p53-mediated apoptosis. Myeloid leukemic cells or murine erythroleukemic cells will undergo apoptosis when p53 is overexpressed and activated (Yonish-Rouach et al., 1991). Treatment of the myeloid cells with interleukin-6 or the erythroid cells with erythopoeitin blocks the p53-mediated apoptic pathway (Yonish-Rouach et al., 1991). In

this case, communication between p53 and a signal transduction pathway results in the reversal of p53-mediated apoptosis.

p53 can also initiate apoptosis in response to the expression of a viral or cellular oncogene or the absence of a critical tumor suppressor gene product retinoblastoma protein (Rb) (El-Deiry et al., 1998a). Rb is the major target of cyclin D/CDK4 for cell cycle regulation (Levine, 1997). The Rb protein regulates E2F-DP transcription factor complexes (E2F-1, -2, and – 3 and DP-1, -2, and –3) which in turn regulate a number of genes including those encoding cyclin E, cyclin A and proliferating cell nuclear antigen (PCNA) required to initiate and propagate the S phase of the cell cycle (Levine, 1997).

The expression of the adenovirus E1A protein in rat embryo fibroblasts has been shown to stabilize and activate p53. The resultant cells die of apoptosis (Debbas and White, 1993). The adenoviruses normally express the E1B-55 kDa protein, which binds to p53 and blocks its transcription factor activity, acts like bcl-2 to block apoptosis downstream of p53 activation (El-Deiry et al., 1998a). The human papilloma virus E7 protein acts like E1A and induces p53-mediated apoptosis. In response, the human papilloma virus genome encodes the E6 oncogene product to bind to p53 and promotes its degradation. Transgenic mice expressing E7 in the retina photoreceptor cells show extensive apoptosis (Howes et al., 1994). The expression of E7 in these same cells but in a p53-/- mouse results in a reduced frequency of apoptosis and an increased frequency of development of retinal tumors (Howes et al., 1994). The E1A and E7 proteins bind to the Rb protein and inactivate its ability to regulate the transcription factor complex, E2F–DP-1 activity (Levine, 1997). Such an unregulated E2F-1 activity in concert with activated p53 results in apoptosis (Levine, 1997). Cells overexpressing E2F-1 and a temperature-

sensitive p53 undergo apoptosis at 32°C but not 37–39°C, where p53 is inactive (Wu and Levine, 1994). Other oncogenes also trigger p53-mediated apoptosis. Cells overexpressing myc and a temperature-sensitive p53 have a temperature-sensitive apoptotic response (Wagner et al., 1994).

Thus, a number of factors affect the decision of a cell to enter a p53-mediated cell cycle arrest or apoptotic pathway. Under conditions in which the DNA or cell cycle spindle machinery is damaged, survival factors for the cells are limiting, or an activated oncogene is forcing the cell into a replicative cycle (E1A, E7, E2F-1, or myc), p53-mediated apoptosis prevails. In this way, cells with unstable genomes (due to DNA or spindle damage) or cells in an abnormal environment (i.e., located in a place with limiting survival factors) with activated oncogenes that commit them to enter the cell cycle are eliminated in a p53-dependent apoptotic event (Levine, 1997). This is most likely the reason why so many cancerous cells select against wild-type p53 function (Levine, 1997).

These observations bring up a number of questions. Clearly, the p53-mediated cell cycle arrest requires p53-dependent transcription and one or more of its downstream genes. Does p53-mediated apoptosis require the transcriptional activity of p53? Furthermore, how does p53 sense the presence of an activated oncogene in a cell (or a pathway activated by an oncogene?). A number of experiments addressing the first question, concerning the need for p53-mediated transcription to induce apoptosis, have concluded that both a p53-mediated transcriptional activity and a p53 activity not requiring transcription can play a role in apoptosis, and the choice depends on the cell type or experimental situation (Levine, 1997; El-Deiry et al., 1998a). In several cell

types, p53-mediated apoptosis initiated by DNA damage occurs in the presence of actinomycin D or cycloheximide, which block RNA or protein synthesis (Caelles et al., 1994). Similarly, a temperature-sensitive p53 in a cell with an activated myc induces apoptosis normally at 32°C in the presence of cycloheximide (Wagner et al., 1994). It has been shown clearly that the transcriptional activation or the translation of p53- regulated gene products are not required in these cases for apoptosis (Caelles et al., 1994; Wagner et al., 1994). Similarly, the introduction of a p53 cDNA fragment that encodes amino acid residues 1–214 into HeLa cells induces apoptosis (Haupt et al., 1995). This fragment fails to bind to DNA or act as a transcription factor. Similarly, p53 with mutations in the transactivating domain (residues 22 and 23) that is unable to activate transcription still induces apoptosis in HeLa cells (Haupt et al., 1995). In contrast, the expression of E1A in baby rat kidney cells or mouse cells failed to induce apoptosis when p53 contained mutations that alter p53-specific DNA binding or transcriptional activation (Sabbatini et al., 1995). Indeed, the mutant p53 that produces amino acid residues 1–214 induced apoptosis in HeLa cells, but failed to induce apoptosis in another cell type, a human lung carcinoma, cell line H1299 (Haupt et al., 1995). Thus, it has been suggested that p53 may use transcriptional activation or direct protein signaling (protein–protein interactions or some other activity) or both to initiate apoptosis (Levine, 1997).

There are a number of genes that are regulated by p53 (in at least some cell types). The earliest mediators of p53-dependent apoptosis to be identified were pro-apoptotic bax gene, p53 induced genes (PIGs), insulin growth factor-binding protein-3 (IGF-BP3) and others (Polyak, et al., 1997; El-Deiry et al., 1998b; Shen and White, 2001). It is clear that no one target of p53 will be sufficient to recapitulate the p53-

dependent apoptosis phenotype under all physiological conditions. However, it is established that Bax and IGF-BP3 can be directly regulated by p53 and can influence the decision to commit to apoptosis (Miyashita and Reed, 1995; Buckbinder et al., 1995). Overexpression of the anti-apoptotic protein Bcl-x (or the adenovirus E1B-19 KDa protein) can block p53-mediated apoptosis (El-Deiry et al., 1998b; Miyashita and Reed, 1995). Bax binds to Bcl-x and antagonizes its ability to block apoptosis, so a p53-dependent Bax synthesis could tip the scales toward apoptosis (Levine, 1997). The mechanism of action of the Bcl2–Bax family of gene products remains unclear. Another p53-regulated gene product that could affect growth regulation is the insulin-like growth factor–binding protein-3 (IGF-BP3) (Buckbinder et al., 1995). IGF-BP3 blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor. Thus, the blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells (Buckbinder et al., 1995). It has been reported that the Fas/APO-1 gene may also be regulated by p53 (Owen-Scaub et al., 1995). The Fas receptor (also known as APO-1 is a potent inducer of apoptosis in hematopoietic or liver cells exposed to Fas ligand (Nagata, 1997). p53 responsive elements have not yet been demonstrated in the Fas/APO-1 gene and so proof that it is truly a p53-regulated gene remains to be provided (Levine, 1997; Wang et al., 1999; Shen and White, 2001).

1.4. Current problems and objectives

As outlined in the above discussion, the signaling pathways that regulate protein kinase CK2 have been elusive since the discovery of this highly conserved and ubiquitous protein-serine/threonine kinase over 45 years ago. Furthermore, the tumor suppressor p53 is one of the key molecules involved in maintaining stability of the genome, through cell cycle arrest and apoptosis in response to genotoxic stress. p53 has previously been shown to be associated with protein kinase CK2, and undergo phosphorylation on Ser-392. However, the mechanism by which Ser-392 is regulated, under circumstances of cellular stress remain to be elucidated.

The goals of this project were:

1. To investigate whether stress-activated signaling regulate protein kinase CK2, and uncover the mechanisms that underlie such regulation.
2. To characterize the physiological regulation and functional consequence of p53 phosphorylation at Ser-392 by CK2 in response to cellular stress especially in the event of mitotic crises in cancer cell cycle.

2. MATERIAL AND METHODS

2.1. Cell Culture and Lysates Preparation.

Human cervical carcinoma HeLa cells were obtained from ATCC and human colon tumor HCT116 (p53 +/+ and p53 -/-, kindly donated by Dr. B. Vogelstein (Bunz et al., 1998). Cells were grown for 3 days until near confluency at 37°C and 5% CO_2 on 175 cm^2 flasks in 10% fetal calf serum (FCS) supplemented DMEM and McCoy's medium (GIBCO), respectively. Cells were split at 5x 10^5 cells /10 cm^2 dish 24 hour prior to treatment. For cell stimulation, HeLa cells were starved with 0.5% FCS for 15-20 h before exposure to stimuli. Starved cells were incubated with 10 μM SB203580 (Calbiochem) for 30 min prior to addition of 10 μg/ml anisomycin (Sigma) for 30 min, 50 μM arsenite (Sigma) for 15 min, or 20 ng/ml TNFα for 15 min. Adherent cells were washed twice with ice-cold, phosphate-buffered saline (PBS), scraped, then lysed in 50 mM Hepes-NaOH (pH 7.2), 100 mM NaCl, 1 mM EGTA, and 20 mM NaF in Buffer A (1 mM sodium orthovanadate, 1% aprotinin, 1 mM phenylmethylsulfonylfluoride, 1 μM pepstatin, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 1% Non-idet P-40). Lysates were subjected to brief sonication for 2 x 10 s, centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were collected and stored frozen prior to use. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

In some experiments, anisomycin-treated cells were lysed with ice-cold 100 mM Tris-HCl (pH 8), 100 mM NaCl, 50 mM $MgCl_2$, 0.3 mM EDTA, 0.5 mM $ZnCl_2$ and 1%

Nonidet P-40. After sonication for 2 x 10 s, the lysates were centrifuged at 14,000 rpm for 5 min at 4°C and the supernatant was incubated with alkaline phosphatase (Calbiochem) (0.5 U/μg) for 2 h at 37°C. Ten volumes of ice-cold Buffer B (10 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 2 mM EDTA, 5 mM EGTA and 2 mM sodium orthovanadate) were added and the sample was subjected to MonoQ anion-exchange chromatography.

2.2. Cell Synchronization

For cell synchronization at G1/S phase, HeLa cells were treated with double thymidine block (two 16 h periods of exposure to 2 mM thymidine separated by a 10 h exposure without thymidine). The cells were then rinsed twice with phosphate buffer saline (PBS) and cultured in complete growth medium. Nocodazole (100-400 ng/ml) was added to either exponentially growing cells for the indicated times or to G1/S synchronized cells for 10 h. In some experiments HeLa cells were cultured in DMEM containing FCS (0.5%) for 30 h to synchronize cells in G0 phase, fresh DMEM supplemented with 10% FCS was then added to induce the cells to re-enter the cell cycle. After 18 h, nocodazole (400 ng/ml) was added before cells entered M phase, as described (Takenaka et al., 1998). Mitotic cells were collected by mechanical shake off, 9 h after release from thymidine block (G1/S) or 22-26 h from G0 release (Takenaka et al., 1998). In some experiments, the potent and specific CK2 or p38 MAPK inhibitors; 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (20 μM) and SB203580 (10 μM) (Calbiochem) respectively, were used 2 h prior to the addition of nocodazole.

2.3. MonoQ Chromatography and Immunoprecipitation.

Cell lysates (2 mg protein) were diluted 1:10 in Buffer B and loaded onto a MonoQ (1-ml; Pharmacia) column at 4°C. The anion-exchange column was eluted with a 20 ml linear gradient of 0-0.8 M NaCl in Buffer B and 0.5 ml fractions were collected at flow rate of 1ml/min.

Immunoprecipitations were performed with a rabbit polyclonal antibody for p38α (Santa Cruz Biotechnology Cat. # SC-535-G), and a mouse monoclonal antibody for CK2β (kindly donated by O.-G. Issinger, Biokemisk Institut, Odense, Denmark). Cell extracts (0.5 mg) were incubated with 4 μg of the appropriate antibody and 20 μl of protein A-Sepharose in the presence of 0.1% SDS for 3 h at 4°C. The beads were washed four times with 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl in Buffer A.

2.4. CK2 Phosphotransferase Activity Assays

CK2 phosphotransferase activity following Mono Q column chromatography was assayed when 5 μl of MonoQ fractions were incubated in a final volume of 25 μl with 5 μg of partially phosphorylated casein or the specific CK2 substrate RRADDSDDDDD and 100 μM [γ—^{32}P]ATP (2.5 μCi/assay in Buffer C (12 mM MOPS (pH 7.2), and 15 mM $MgCl_2$) for 10 or 15 min at 30 °C. These and other CK2 assays were performed in the absence and presence of either 20 μg/ml heparin (Sigma), 20 μM DRB (Sigma) or 10 μM SB203580 (CalBiochem). The phosphorylation of casein was quantitated by spotting 20 μl on to a 1.5 cm^2 piece of Whatman P-81 phosphocellulose paper. The papers were

washed extensively in 1% (w/v) phosphoric acid, transferred into 6-ml plastic vials containing 0.5 ml of Ecolume (ICN) scintillation fluid, and the incorporated radioactivity was measured in a Wallac (LKB) scintillation counter.

Recombinant CK2 holoenzyme (Calbiochem) (1 μg) was incubated with 250 ng of the wild-type glutathione-S-transferase (GST)-p38α (Kinetek Pharmaceuticals, Inc.) or wild-type GST-Erk1 (Kinetek Pharmaceuticals, Inc.) in a final volume of 25 μl with 10 μCi of 100 μM [γ32—P]ATP in Buffer C for 15 min at 30°C. Reactions were terminated by the addition of SDS-PAGE sample buffer (Laemmli, 1970). After SDS-PAGE, radioactivity incorporated into CK2 was detected by autoradiography.

2.5. Immune-Complex Kinase Assays

Immunoprecipitations were performed by incubating 500 μg of whole lysate with 4 μg of CK2α monoclonal (kindly donated by Dr. Olaf Issinger), -cdc2 polyclonal (donated by Kinetek Pharmaceuticals, Inc) or -p38α (Santa Cruz Biotechnology) polyclonal antibody and 20 μl of protein A-Sepharose (preblocked with 1% BSA) for 3-4 h at 4°C. The beads were washed four times with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40 and the aforementioned protease and phosphatase inhibitors. The immunoprecipitated beads were washed one additional time with buffer A (12 mM MOPS (pH 7.2) and 15 mM $MgCl_2$), and incubated with either 5 μg of casein of the specific CK2 substrate RRADDSDDDDD, the p38 MAP kinase substrate ATF2, or with the cdc2 substrate H1-histone (Calbiochem) in a final volume of 25 μl with 5 μCi or 10 μCi of 100 μM [γ-^{32}P] ATP in Buffer A, for 10 or 15 min at 30°C. Reactions were

stopped by either spotting 20 µl onto 1.5 cm^2 piece of Whatman P-81 phosphocellulose paper and the incorporated radioactivity was measured in a Wallec (LKB) scintillation counter, or by the addition of SDS-PAGE sample buffer. After boiling, the samples were subjected to SDS-PAGE for autoradiography with X-ray film or by immunoblotting analysis.

2.6. Immunoblotting

Western blotting was carried out with 0.1 to 1 µg/ml of the rabbit polyclonal antibodies: phospho-p38α (New England Biolabs, Cat. # 9211S), p38α, (StressGen Biotechnologies, Cat. # KAP-MA009), CK2α-III (Hagemann et al., 1997), and CK2β (Kinetek Pharmaceuticals, Inc.), mouse monoclonal cyclin B1 (Pharmingen, Cat. # 14551A), p53 (StressGen, Cat. # KAM-CC002), phospho-p53 Ser-392, phospho-cdc2 (New England Biolabs, Cat. # 9281S, 9110S, respectively), mouse monoclonal CK2α (kindly donated by Dr. Olaf-Georg Issinger), rabbit polyclonal anti-p34 cdc2 (Kinetek Pharmaceuticals, Inc), or p38α (Santa-Cruz Biotechnology) antibodies, were also used.

2.7. *In vitro* Binding assay

Approximately 2 µg of recombinant CK2 holoenzyme (Calbiochem) were mixed with 400 ng of either GST-p38, GST or GST-Erk1. The recombinant proteins were incubated for 1 h at 4°C in Buffer D [12.5 mM MOPS (pH 7.2), 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, and 0.5 mM NaF], and the GST was

captured by addition of 25 µl of a 1:1 slurry of glutathione-Sepharose 4B beads for another hour at 4°C. In some experiments, 20 µM SB203580, 20 µg/ml heparin or 20 µM DRB was incubated with the GST-p38α or the recombinant CK2 for 15 min at 30°C prior to the addition of the recombinant CK2 or GST-p38, respectively. The beads were washed 3 times with Buffer D containing 0.1% NP-40 and the bound proteins were eluted by addition of SDS-PAGE sample buffer. After boiling, the samples were subjected to SDS-PAGE on 12% acrylamide gels and immunoblotted with either CK2α–III or CK2β antibodies.

2.8. Oligonucleotide Transfection

Cells were grown in medium containing FCS (0.5%) for 24 h prior to transfection, washed twice with PBS and supplemented with fresh medium containing 10% FCS. CK2α-α' antisense or sense oligonucleotide (2 µg/ml) was transfected into cells using Effectene (Qiagen) according to the manufacturer's protocol. CK2α-α' antisense and sense were designed as previously described (Ulloa et al., 1993), with a modification in which the entire oligonucleotides were phosphorothioated; antisense (5' to 3'): GTA ATC ATC TTG ATT ACC CCA or sense (5' to 3'): TGG GGT AAT CAA GAT GAT TAC. Cells were incubated with the oligonucleotides for 18-24 h prior to any either treatments.

2.9. Flow Cytometry

Cells were trypsinized, washed with PBS and fixed with 70% ethanol at the indicated time points then stored at –20°C. The fixed cells were then stained with propidium iodide (50 μg/ml) with RNase (10 μg/ml). The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (Becton Dickinson Instruments). The forward and side scatter gates were set to exclude any dead cells from the analysis; 10,000 events within this gate were acquired per sample.

2.10. Mitotic Index

Cells were collected by incubation with trypsin containing EDTA, centrifuged, counted for normalization and then fixed in a solution containing 3.7% formaldehyde, 0.5% NP-40 and Hoescht 33258 (10 μg/mL) in PBS. Nuclei were visualized by fluorescent microscopy. Nuclei with condensed, evenly stained chromosomes were scored as mitotic (Bunz, et al., 1998). At least 600 cells were counted for each determination.

2.11. Apoptosis Assay

Cells were collected at the indicated time points by incubation with trypsin-EDTA, centrifuged and washed twice with ice-cold PBS, then stained with Annexin-V FITC and propidium iodide (PI) according to the manufacturer's protocol (Pharmingen).

Cells were analyzed using the CellQuest software on a Becton Dickinson FACScan and 10,000 events were acquired per sample.

2.12. Immunofluorescence

Cells were grown on coverslips (~70% confluent) and fixed at -20°C with 100% methanol overnight. After being blocked in PBS containing 4% bovine serum albumin, cells were doubly stained with mouse monoclonal anti-CK2α and rabbit polyclonal anti-γ-tubulin (Sigma) or mouse monoclonal anti-p38α (Transduction Laboratories) and anti-γ-tubulin for 3 h. Cells were washed six times with PBS containing 0.1% Triton X-100 and incubated for 1 h with goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and goat anti-rabbit IgG conjugated to rhodamine (both at 1:100 dilution, Jackson Laboratory). After six washes in PBS, cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (0.1 μg/mL in PBS, Molecular Probes). Coverslips were mounted on glass slides with anti-fade (Molecular Probes) and analyzed via fluorescent microscopy.

3. STRESS INDUCED ACTIVATION OF PROTEIN KINASE CK2 BY DIRECT INTERACTION WITH p38 MAP KINASE

3.1. Rationale and Hypothesis

The mechanisms that regulate CK2 phosphotransferase have been elusive since it has not been shown to be regulated by any of the well-known second messenger molecules. However, its activity can be affected by a number of compounds that may also be involved in p38 MAP kinase activation such as tumor necrosis factor (TNF), IGF-1, and insulin (Pinna and Meggio, 1997; Widmann, 1999). p38 MAP kinase has been also shown to play multiple roles a role in cell growth and division through unknown targets (Nebreda and Porras, 2000). Furthermore, preliminary data from our laboratory showed constitutive elevation of p38α and CK2 expression levels in many primary breast, colon and lung tumors. Using gel-based protein renaturation method for protein kinase activity in crude extracts from the former human solid tumors, the major autophosphorylated protein kinase was identified as CK2 (B. Salh and S. Pelech, unpublished observations).

I therefore tested whether stress-activated p38 MAP kinase might participate in CK2 regulation.

3.2. RESULTS

3.2.1. Stress factor-induced activation of CK2 and p38 MAP kinase

To explore the role of stress stimuli in the regulation of CK2, we investigated the effect of various stress stimuli, the protein synthesis inhibitor; anisomycin, the mitochondrial oxidation inhibitor; arsenite and the inflammatory cytokine; TNF-α, that have been shown to induce p38-stress signaling pathway (Hazzalin et al., 1998; Bernstam and Nriagu, 2000; Liu et al., 2000; Ono and Han, 2000; Namgung and Xia, 2001). Serum-deprived HeLa cells were treated with these agents and the casein phosphotransferase activity of CK2 was assessed following MonoQ fast protein liquid chromatography of the cell lysates. Anisomycin treatment caused an 8-fold activation of CK2, which eluted with 0.6 M NaCl in primarily fraction numbers 18 and 19 (Fig. 2A). This was confirmed by the sensitivity of the casein phosphotransferase activity peak to the CK2 inhibitor heparin (Fig. 2A), and the elution of the α, α' and β subunits of CK2 in these MonoQ fractions as revealed by immunoblotting analysis (Fig. 3 A, and D). Exposure of the serum-starved cells to the mitochondrial oxidation inhibitor arsenite or TNF-α for 15 min was also observed to stimulate CK2 activity by 3- to 4-fold (Fig. 2B). Furthermore, using a highly specific CK2 substrate peptide (RRADDSDDDDD), cytosolic extracts from HeLa cells treated with arsenite or anisomysin, respectively, contained 6.33 ± 0.06 and 7.44 ± 0.03 (mean ± S.E., $n = 3$) fold more phospho-transferase activity than was detectable with cytosol from non-treated cells (data not shown).

Under the same conditions that activated CK2, anisomycin, arsenite and TNF-α also produced marked increases in the phosphorylation of the p38 MAP kinase as determined by immunoblotting with an antibody specific for the activating phosphorylation site of p38 isoforms (Fig. 2C).

To assess whether p38 MAP kinase mediated the stress factors-induced activation of CK2, we took advantage of the specific p38 inhibitor SB203580. Treatment of the serum-deprived HeLa cells with SB203580 for 30 min prior to their exposure to anisomycin, arsenite or TNFα abrogated the ability of each of these agents to stimulate CK2 (Fig. 2 A and B).

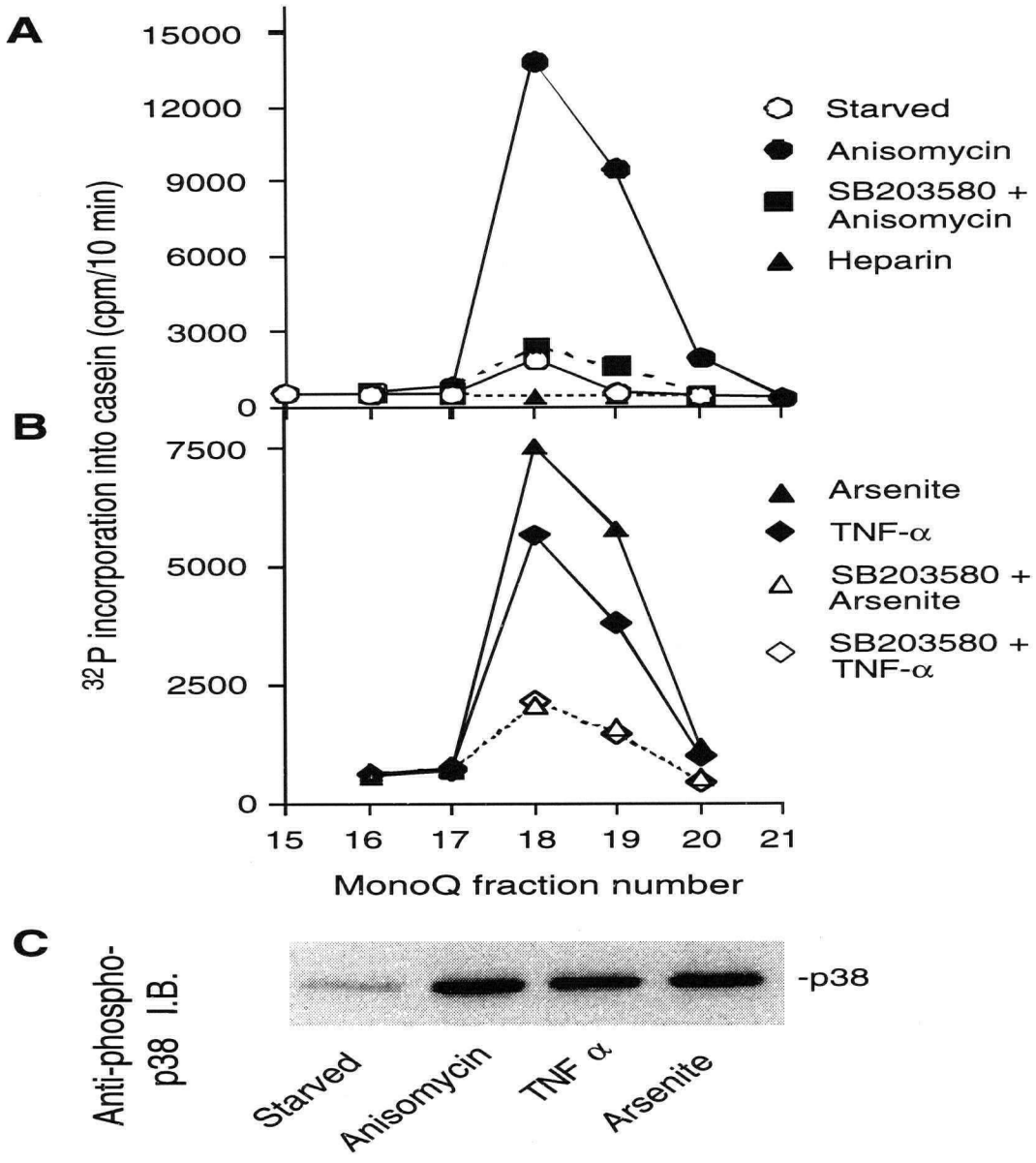

Figure 2: **Stress factor induced activation of CK2 and p38 MAP kinase.** **A, B,** HeLa cells were serum deprived (0.5%) for 18 hours and then treated with either anisomycin 10 μg/ml (30 min), arsenite 50 μg/ml (15 min) or TNFα 20 ng/ml (15 min) resulted in the activation of CK2, which elutes in fractions 18 and 19 following MonoQ chromatography of the cell lysates. These activations could be blocked by pretreatment of the cells with the p38 MAP kinase inhibitor SB203580 (10 μM) or during the CK2 assay by heparin (20 μg/ml). **C,** Immunoblotting of cell lysates with an antibody specific for the phosphorylated form of p38 MAP kinase indicated that anisomycin, arsenite or TNFα activated p38 in HeLa cells. Results are representative of at least three experiments.

3.2.2. Phosphorylated and active p38 MAP kinase coelutes with CK2 from Mono Q

As the anisomycin treatment consistently elicited the most striking activation of CK2, we exploited this agent to further explore the mechanism for the CK2 stimulation. There were no differences in the intensity of immunostaining of the α, α' and β subunits of CK2 in serum-deprived, anisomycin and SB203580-treated cells (Fig. 3A to F). Consequently, the anisomycin appeared to increase the specific enzyme activity of CK2 without alteration of the α, α'/β subunit ratio or the total amount of CK2 protein. There was also no apparent reduction in the mobilities of the CK2 subunits following SDS-polyacrylamide gel electrophoresis of the MonoQ fractionated lysates from cells exposed to anisomycin. Such retardation of CK2 subunits usually reflects their increased phosphorylation.

CK2 subunits have been reported to interact with many other proteins, including the protein kinases Mos, Raf A and Rsk, which bind to CK2β (Guerra et al., 1999). Therefore, we examined whether a p38 MAP kinase might directly associate with CK2. The peak of immunoreactivity of the α-isoform of p38 was found to coelute with CK2 following MonoQ fractionation in fractions 18-20, and this was most pronounced in lysates from anisomycin-treated cells (Fig. 3G, and H). The densitometric analysis showed a 1.6 fold increse of p38 protein eluted in fractions 18-20 in anisomycin-trated cells (results not shown). In contrast, the amount of p38α coeluting with CK2 was substantially reduced in extracts from cells treated with both anisomycin and SB203580, and a large portion of the MAP kinase failed to bind to the MonoQ column and eluted in wash through fractions 4 to 7 (Fig. 3I). An even more dramatic redistribution of p38α

away from CK2 was produced when the lysates from cells exposed to anisomycin were incubated with alkaline phosphatase to dephosphorylate the p38α prior to MonoQ chromatography (Fig. 3J). CK2 subunits were not detectable by immunoblotting in MonoQ fractions 4 to 7 neither before nor after alkaline phosphatase treatment and it eluted in fractions 18 to 20 without any change in protein levels (results not shown). Furthermore, the alkaline phosphatase treatment abolished the anisomycin-induced activation of CK2 (data not shown). The implication of these experiments was that p38α MAP kinase must be in an active conformation in order to maximally bind and activate CK2.

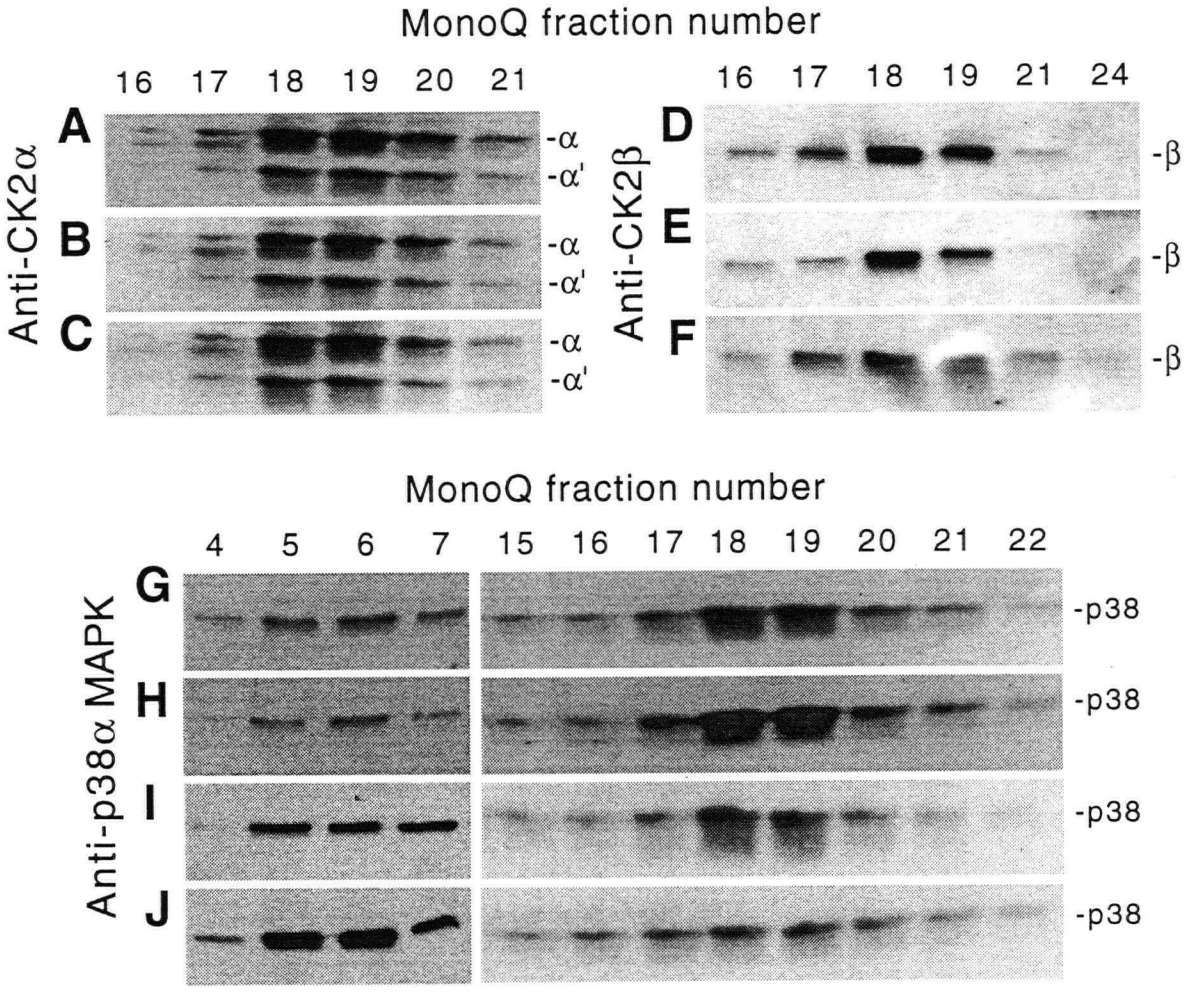

Figure 3: **Phosphorylated and active p38 MAP kinase coelutes with CK2 from MonoQ**. Following MonoQ fractionation, lysates from HeLa cells were immunoblotted with anti-CK2-III (recognizes α/α' subunits), CK2β or p38α antibody. Figure shows that lysates from untreated (**A**,**D**, and **G**) or those exposed to anisomycin in the absence (B,E,H,J) and presence of SB203580 (**C**,**F**, and **I**), there were no detectable changes in the distribution or amount of immunoreactive CK2 α- (**A** to **C**) and β- subunits (**D** to **F**). p38 eluted principally in the wash through and CK2-containing fractions. Following anisomycin treatment of cells, the amount of p38 associated with CK2 was increased (**H**) as compared to untreated cells (**G**) or SB203580-pretreated cells subsequently exposed to anisomycin (**I**). **J**, following incubation of lysates from ansiomycin-treated cells with alkaline phosphatase (0.5 units/μg), most p38 eluted in the wash through fractions from MonoQ.

3.2.3. Active p38 MAP kinase coimmunoprecipitates with CK2

While the preceding data for CK2 and p38α residing in a common complex was only correlative, direct evidence was obtained through coimmunoprecipitation studies. The p38α antibody was used to immunoprecipitate equivalent amounts of p38α from lysates of serum-deprived, anisomycin-, and SB203580 + anisomycin-treated cells (Fig. 4). The casein phosphotransferase activity was approximately 4-fold higher in the immunocomplexes from anisomycin-treated as compared to untreated cells, and this was reduced to less than a 2-fold stimulation by the SB203580 (Fig. 4). These immunocomplexes with p38α antibody were shown to contain both the α and β subunits of CK2 by immunoblotting, and their presence was increased in the immunocomplexes from anisomycin-treated cells (Fig. 5B and C). Reciprocally, in immunocomplexes obtained with an antibody specific for CK2β, p38α was largely immunodetected in samples from anisomycin-treated cells, with little if any evident in immunocomplexes from serum-starved or SB203580 + anisomycin -treated cells (Fig. 5 D).

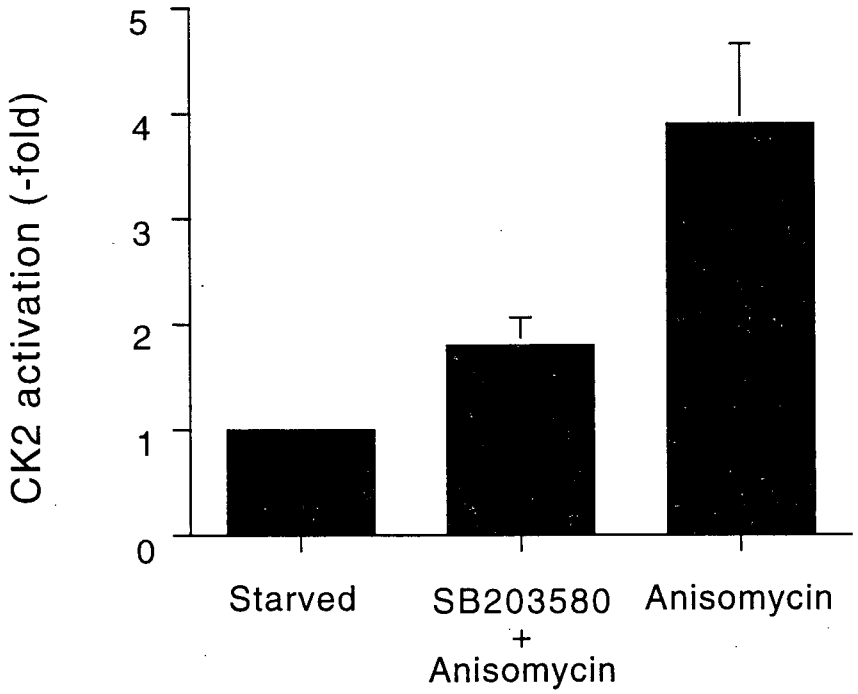

Figure 4: Activation of CK2 in anisomycin-stimulated p38α MAP kinase immunoprecipitates. Lysates of anisomycin-treated HeLa cells (10 μg/ml; 30 min.) were immunoprecipitated with a p38α polyclonal antibody. CK2 kinase assay was determined using casein or RRADDSDDDDD substrate substrate (5 μg). Averages and standard error of the mean of three separate experiments are shown.

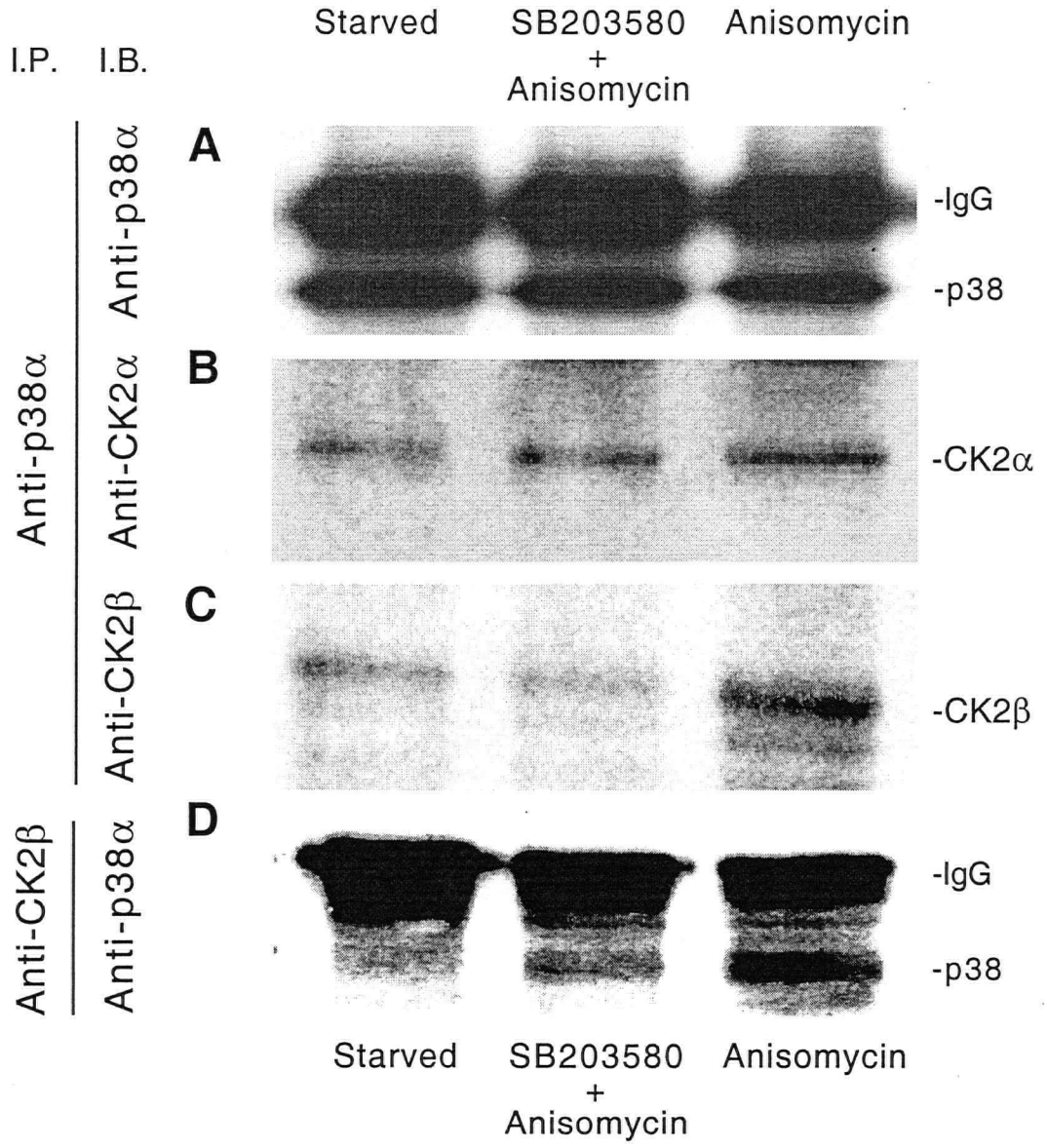

Figure 5: **p38α MAP kinase coimmunoprecipitates with CK2.** Lysates from anisomycin-treated cells were immunoprecipitated with anti-p38α or CK2β antibody and then immunoblotted with anti-p38α, CK2α or CK2β antibody. Immunoprecipitates (**A** to **C**) obtained with a p38α polyclonal antibody from lysates of anisomycin-treated cells featured higher levels of immunoreactive CK2α and CK2β subunits than from untreated or anisomycin and SB203580-treated cells. **D**, Immunoprecipitates obtained with the CK2β antibody from lysates of anisomycin-treated cells displayed higher levels of immunoreactive p38α than from untreated or anisomycin and SB203580-treated cells.

3.2.4. p38α MAP kinase expressed as glutathione S-transferase fusion protein binds to and activates recombinant CK2

Further support for the direct interaction of CK2 and p38α was obtained through the use of recombinant CK2 holoenzyme (CK2α/β) and recombinant p38α expressed as a glutathione S-transferase (GST) fusion protein. A basal activity of the GST-p38 and CK2 were shown when assayed ATF2 or casein substrate, respectively, *in vitro* (results not shown). GST-p38 displayed binding for recombinant CK2 protein (Fig. 6C). The interaction of CK2 for GST-p38α was specific, since no binding of CK2 α was detected when either GST or GST fused to the MAP kinase Erk1 was substituted for GST-p38 α (Fig. 6C). GST-p38 stimulated the casein phosphotransferase activity of recombinant CK2 by 2-fold, and this was sensitive to SB203580 and the CK2 inhibitors heparin and 5,6-dichloro-ribifuranosylbenzimidazole (DRB) (Fig. 6A). When the CK2/GST-p38α complex immobilized on glutathione-Sepharose was incubated with [γ^{32}P]ATP, there was strong phosphorylation of the β- subunit of CK2 (Fig. 6B), but not the α–subunit of CK2 (data not shown). This phosphorylation was abolished when the incubations with [γ-^{32}P]ATP were carried out with SB203580, DRB or heparin. When the presence of CK2α was ascertained by immunoblotting, the DRB and heparin did not dissociate the CK2/GST-p38α complex, whereas SB203580 did (Fig. 6C). These findings indicated that the radiolabelling of CK2β in the presence of GST-p38 was largely due to autophosphorylation, apparently from the stimulation of CK2 catalytic activity. This was further supported by experiments in which the CK2 was preincubated with unlabeled ATP for 15 min to saturate the autophosphorylation sites, and then with [γ-^{32}P]ATP in

the absence and presence of GST-p38α for an additional 10 min. Under these circumstances, there was no enhanced activity of the recombinant CK2 toward casein substrate with the addition of GST-p38α. However, approximately two-fold increases in the casein phosphotransferase activity of CK2 were observed when GST-p38α added in the presence of [γ ^{32}p] ATP alone (Fig. 7). The latter result indicates that p38 MAP kinase is unlikely to be able to phosphorylate CK2 directly.

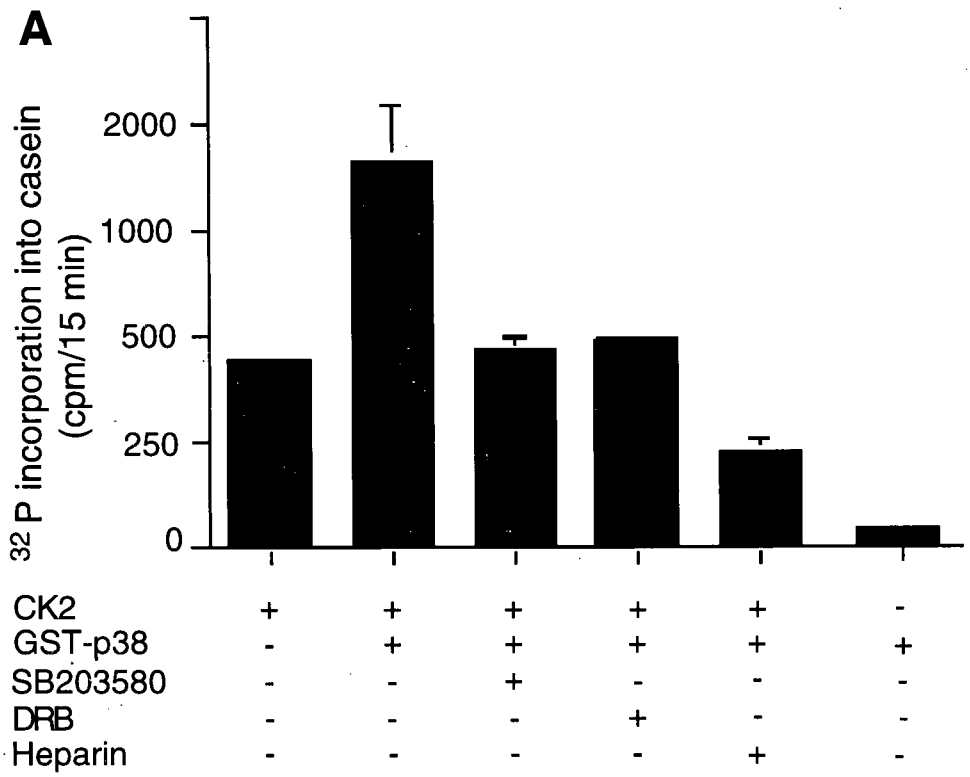

Figure 6 A-C : p38 αMAP kinase expressed as a glutathione S-transferase fusion protein binds to and activates recombinant CK2. **A**, The GST-p38α–stimulated casein phosphotransferase activity of CK2 is inhibited by SB203580, DRB and heparin *in vitro*. Two μg of the recombinant CK2 were incubated with 400 ng of GST-p38 in the presence and absence of SB203580 (20 μM for 15 min), DRB (20 μM for 15 min) or heparin (20 μg/ml for 15 min) for 1 h at 4°C. CK2 kinase activity was measured according to the material and methods using casein as the substrate. Averages and standard error of the mean of three separate experiments are shown.

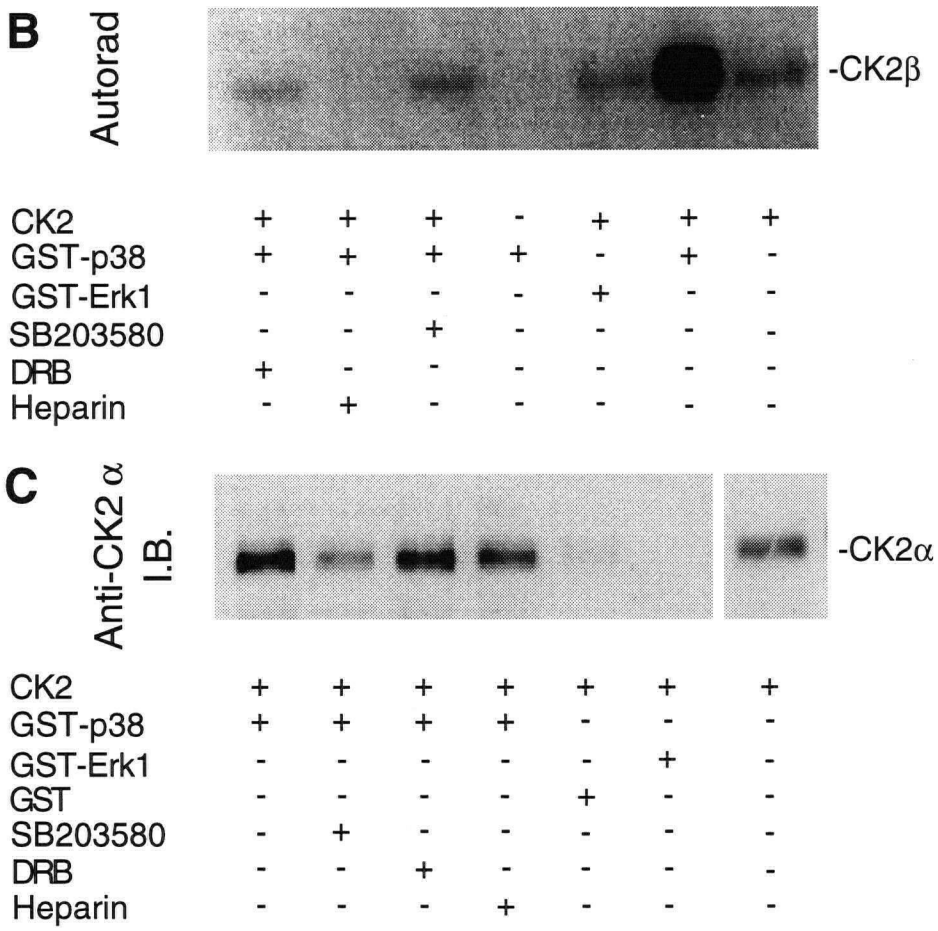

Figure 6 B&C: **B**, The GST-p38 α–stimulated autophosphorylation of CK2β subunit is inhibited by SB203580, DRB and heparin *in vitro*. Two μg of recombinant CK2 were mixed with GST-p38, GST, or GST-ERK1 in the presence and absence of SB203580, DRB or heparin and immobilized on glutathione beads as described in material and methods. Five μg of the CK2 specific substrate RRDDSDDDDD were incubated with the immunoprecipitated beads for 15 min at 30 °C in the presence of [γ-^{32}p] ATP and the reaction was stopped by adding SDS sample buffer. The radioactivity incorporated into CK2 was measured by autoradiography. No detectable CK2α autophosphorylation was observed. **C**, CK2 binds to GST-p38α, but not the Erk1 MAP kinase, and the association is inhibited by SB203580, but not by DRB or heparin. The immunoprecipitates from the above treatments were probed with CK2α antibody. The right most lane shows the migration position of recombinant CK2α.

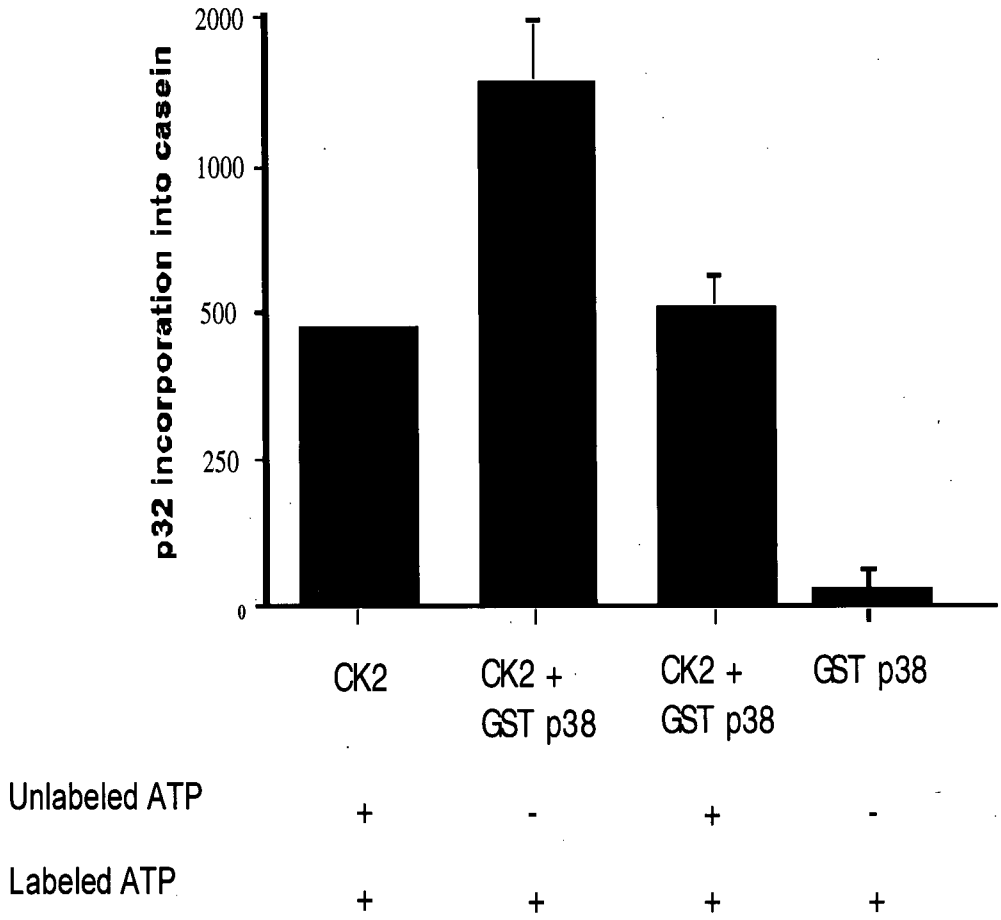

Figure 7: p38 MAP kinase does not phosphorylate CK2 *in vitro*. CK2 was preincubated with unlabeled ATP for 15 min to saturate the autophosphorylation sites, and then with [γ-^{32}P]ATP in the absence and presence of GST-p38α for an additional 10 min, lanes 1 and 3. The activity of CK2 was measured using 5 μg of casein as the substrate. Lanes 2 and 4 show the casein phosphotransferase activity of the recombinant CK2 preincubated with GST-p38 or p38 alone, respectively, in the presence of [γ-^{32}P]ATP alone. Averages and standard error of the mean of three separate experiments are shown.

3.3. Discussion

Protein kinase CK2 has been implicated in the regulation of a wide range of proteins that are important in cell proliferation and differentiation. Here we were able to demonstrate that the stress signaling agents anisomycin, arsenite, and tumor necrosis factor-α stimulate the specific enzyme activity of CK2 in the human cervical carcinoma HeLa cells by up to 8-fold, and this was blocked by the p38α MAP kinase inhibitor, SB203580. Our results clearly demonstrated that p38 phosphorylation is a prerequisite for interaction with CK2 and the pyridinyl imidazole compound SB203580, p38 MAP kinase specific inhibitor, prevented complex formation.

Further characterization of the CK2-p38 interaction was carried out by immunoprecipitation and immunoblotting experiments. We observed increase amounts of the CK2α protein in stress-stimulated cells which was decreased upon SB203580 treatment. However, in examining the immunoblots with anti-CK2β-antibody, it was interesting to observe that CK2β was more abundant in cells stimulated by various stress stimuli and to a smaller extent in starved cells. Similar results were observed in anti-CK2 β immunoprecipitates. Pretreatment with SB203580, samples from anisomycin-treated cells, completely abolished the presence of CK2β protein (Fig. 5). These results indicate that p38 MAP kinase may engage in a complex formation with CK2, perhaps via a protein-protein interaction. This was dependent on the activation and phosphorylation of p38 (Fig. 2-3), since pretreatment with SB203580 and alkaline phosphatase prevented the complex formation. Furthermore, we demonstrated that the p38 MAP kinase was not able to directly phosphorylate CK2 (Fig. 7). Further support for the direct interaction

between CK2 and p38 was obtained through the use of recombinant CK2 and p38. Our results clearly showed that p38 had a great affinity for CK2 *in vitro* that led to increase in CK2 activation by 2 fold and the p38 MAP kinase specific inhibitor, SB203580, disrupted this interaction (Fig. 6). SB203580 was shown to have no effect on the activity of recombinant CK2 alone *in vitro* (data not shown). SB203580 has been reported to affect p38 MAP kinase activity by binding within the ATP pocket at the hinge region that is involved in protein-protein interaction (Gum and Young, 1999). Thus, the p38 inhibitor SB203580 attenuates CK2 activation, perhaps by interfering in the protein-protein interaction between CK2 and p38.

Our data indicate for the first time that following activation of p38 MAP kinase, CK2 is activated by a protein-protein interaction that leads to autophosphorylation of the CK2β subunit (Fig 6B). CK2β has been shown to undergo autophosphorylation when it is assembled with and activated by CK2α (Pinna, 1997; Guerra and Issinger, 1999). Furthermore, CK2β is required for formation of the tetrameric CK2 complex, both negative and positive regulation, specificity and stability of CK2α (Pinna, 1997; Guerra and Issinger, 1999). It is conceivable that the activation of CK2α by the activated p38 may attract or induce the formation of α-β dimerization. This scenario is very attractive since the map of a modeled tertiary structure of the region of α-β link revealed that the CK2β binding site in CK2α is located in the region linking two lobes but on the other side of the activation loop of the kinase domain (Guerra et al., 1998; Niefind et al., 1998)□. Thus it does not hinder the access of substrates to the kinase and is proximal to the ATP binding region. Based on the finding that the dimeric form of the C-terminal β fragment is mainly responsible for α-β interaction, and it binds more efficiently than its

monomeric form, this indicates that either β dimerization is a prerequisite for tight binding of α, or each of the catalytic subunit needs to make contact for high affinity association (Guerra et al., 1998; Guerra et al., 1999). It is possible that p38 binding with β may promote a conformational change that facilitates subsequent binding of the catalytic subunit of CK2 that is already bound to p38.

Collectively, our results support a model in which phosphorylated p38α is able to engage CK2 in a complex through direct protein-protein interaction. It would appear that an active conformation of p38α is needed for binding, since the SB203580 prevented complex formation. Perhaps, through an allosteric effect exerted by active p38α, CK2 may become stimulated to phosphorylate other targets. It is unclear if the increased autophosphorylation of CK2 may be sufficient to preserve the state of activation of CK2 after p38α is dissociated.

4. ACTIVATION AND LOCALIZATION OF PROTEIN KINASE CK2 IN NOCODAZOLE-INDUCED MITOTIC ARREST

4.1. Rationale and Hypothesis

The above results have implicated protein kinase CK2 in mediation of stress signaling downstream of p38 MAP kinase. Takenaka et al. (1998) have shown that the p38 MAP kinase, but not p42/p44 MAPK or JNK was activated in the mitotic arrest by disruption of the spindle with nocodazole. This study indicated that p38 MAP kinase functions as a component of the spindle assembly checkpoint in the somatic cell cycle and contributes in mitotic stress possibly modulating CDK-1 function. In view of the association of p38 with CK2 in the previous chapter, I therefore explored whether CK2 participated in the regulation of spindle assembly.

4.2. Results

4.2.1. Depletion or inhibition of CK2 compromises mitotic arrest

To investigate whether CK2 is required for mitotic checkpoint function, we employed antisense to the alpha subunit to deplete the protein kinase. We transfected HeLa cells with either CK2 (α/α' subunits) antisense (AS) or sense (S) oligodeoxyribonucleotide. The expected depletion of CK2 upon AS oligonucleotide treatment was confirmed by assaying the intracellular protein level of CK2 α/α' subunits by immunoblotting (Fig. 8B). The densitometric analysis showed 80% reduction of CK2 protein from Fig. 8B in As-treated cells (results not shown). Furthermore, we showed negligible activity of CK2 in cells transfected with CK2 antisense (data not shown). Previous studies, using the same antisense oligonucleotide, have demonstrated not only complete depletion of the catalytic CK2 α/α' subunits (Ulloa et al., 1993), but also inhibition of sorting of the regulatory β subunit, preventing its translocation to the nuclei and dimerization with α subunits (Ulloa et al., 1994). Cells were then synchronized at G0/G1 phase and released into the cell cycle, by the addition of serum-containing medium (10%) as previously described (Takenaka et al., 1998). Nocodazole, a drug that disrupts the mitotic spindle by inhibiting microtubule polymerization, was added before cells entered the mitotic (M) phase. After 9-10 hours, microscopic examination of transfected cells with either S oligonucleotide or buffer alone revealed that many of them became rounded, indicative of cells arrested in mitosis (Fig. 8A). In contrast, cells transfected with AS showed fewer rounded cells. We then measured the percentage of

cells in mitosis (mitotic index, MI) after nocodazole treatment. The average MI of cells with either S-treated or buffer was 63% (1008 of 1600 cells), whereas the MI of cells transfected with AS was 11% (176 of 1600) (Fig. 9). These results indicated that CK2 depletion was responsible for a reduction in the mitotic cell fractions induced by nocodazole. Similar results were observed using the CK2 specific inhibitor DRB that was added two hours prior to nocodazole exposure (Fig. 9).

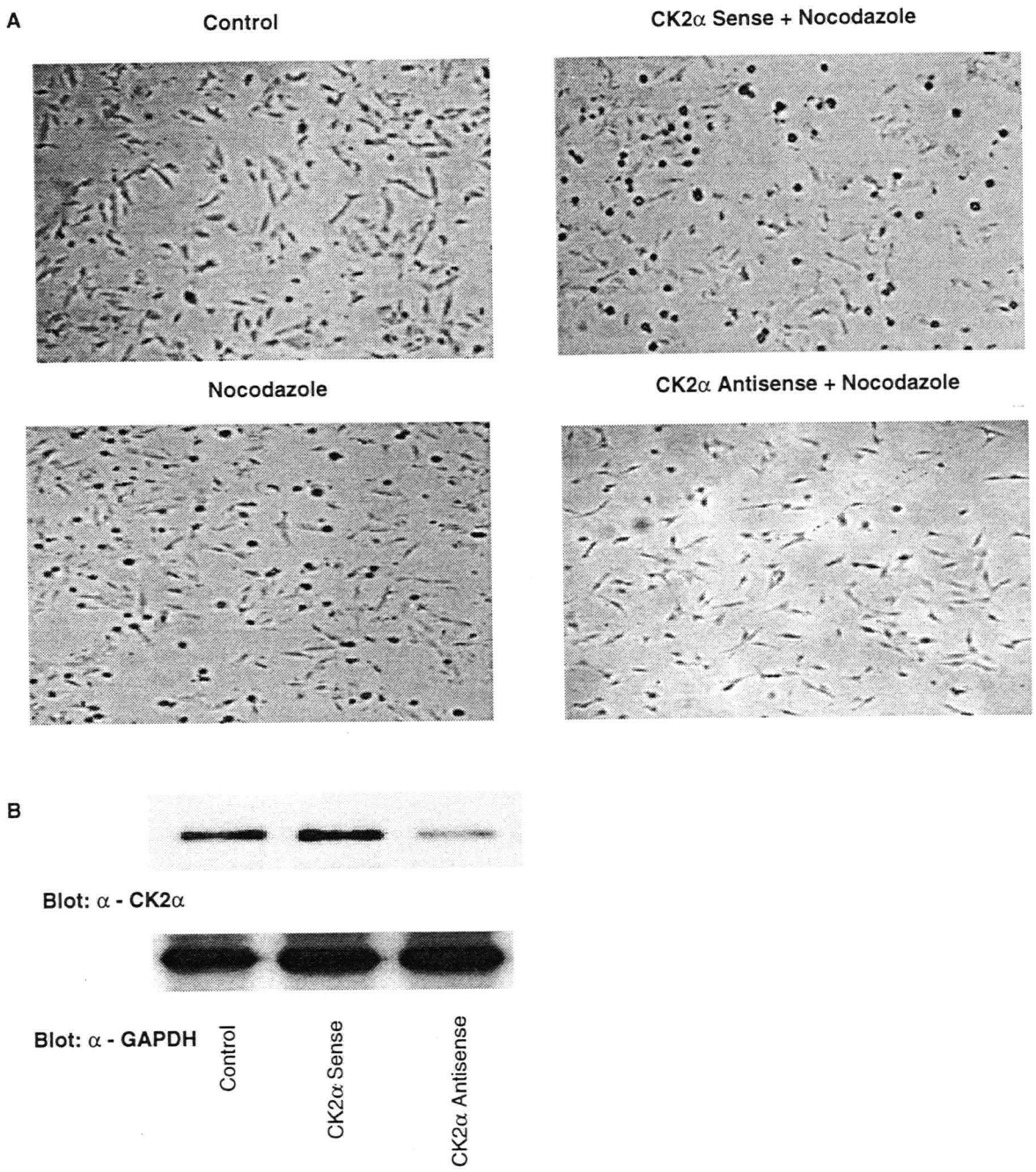

Figure 8: Depletion of CK2 compromises mitotic arrest. A, HeLa cells, treated with CK2 sense, antisense oligonucleotides or left untreated (control), were synchronized at G0/G1 phase in serum deprived medium (0.5%) for 30 hours. Cells were then released into the cell cycle by addition of serum (10%). After 18 hours, cells exposed to nocodazole (400 ng/ml) for 12 hours before being photographed. **B**, Depletion of the endogenous CK2α in HeLa cells transfected with CK2α-α' antisense. Protein extracts were subjected to protein immunoblot analysis with monoclonal anti-CK2α antibody. Lower Panel shows equal amount of proteins loaded by immunoblotting with polyclonal α-GAPDH antibody.

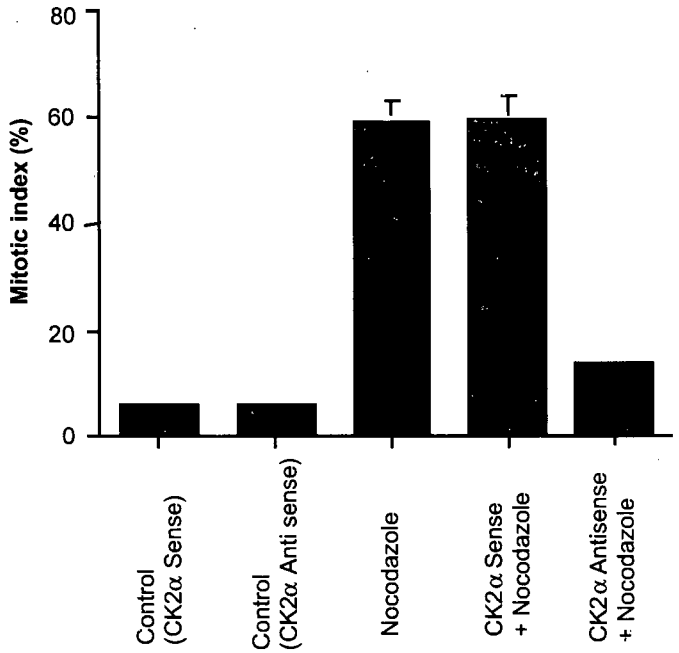

Figure 9: **Failure of HeLa cells to undergo mitotic arrest in the event of CK2 depletion or inhibition.** HeLa cells, treated with CK2 sense, antisense or left untreated, were deprived of serum (0.5%) for 30 hours and released into the cell cycle by addition of serum. After 18 hours, cells exposed to nocodazole (400 ng/ml) for 12 hours. The percentage of cells in mitosis (mitotic index) were measured. Nuclei with condensed, evenly stained chromosomes with Hoescht 33258 were scored as mitotic cells. At least 600 cells were visualized in three independent experiments. Averages and standard error of the mean of three separate experiments are shown.

4.2.2. CK2 functions as a component of the spindle assembly checkpoint

The spindle assembly checkpoint is the mechanism that prevents the onset of anaphase in cells that have defects in spindle assembly or chromosomal alignment on the spindle (Li and Nicklas, 1995). As I indicated above, it has been demonstrated that inhibition of p38 MAP kinase activity, in response to spindle disruption, compromises the spindle assembly checkpoint (Takenaka et al., 1998). Based upon our earlier observations that p38 MAP kinase and CK2 interact directly (Chapter 3), we therefore tested whether CK2 might also participate in the spindle assembly checkpoint of the somatic cell cycle. Transfected HeLa cells with S, AS, or left untreated were synchronized at G1/S phase boundary by a double thymidine block and released into the cell cycle. Cells were then either treated with nocodazole for 10 hours or released without treatment for 9 hours during which the mitotic cells were collected by mechanical shake off. Microscopic examination (results not shown) and lysates from shake off and nocodazole-treated cells contained kinase activity toward histone H1, a measure of CDK-1 consistent with the released cells from G1/S block being in M phase (Fig. 10A). A dramatic increase in CDK-1 activity was observed in nocodazole-treated cells, indicative of mitotic arrest (Fig. 10A and B). These activities were substantially suppressed in AS-transfected cells after nocodazole exposure. Thus, the activation of mitotic spindle assembly checkpoint by the microtubule inhibitor, nocodazole, was compromised by depletion of CK2. In agreement with Takenaka et al. (1998), our results indicate that the activation of CDK-1 by nocodazole was also suppressed in cells pre-treated with the p38 MAP kinase specific inhibitor SB203580 (Fig. 9A and B).

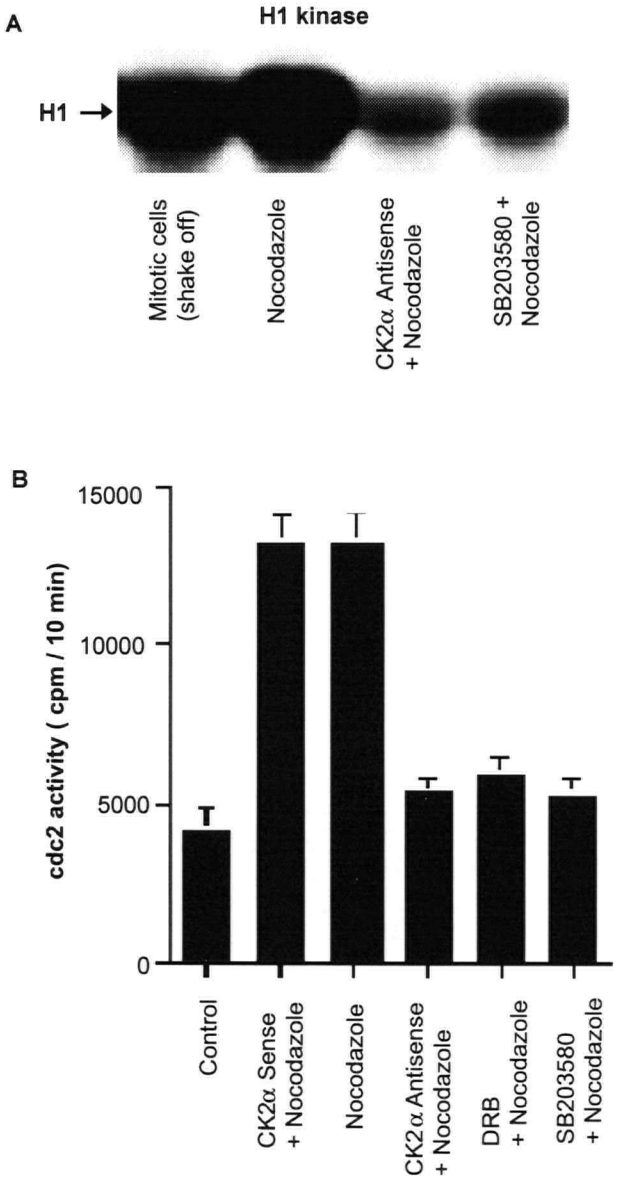

Figure 10: A, Suppression of nocodazole-induced MPF activity by either CK2 antisense or a specific inhibitor of p38. HeLa cells were synchronized at G1-S by double thymidine block and either collected (control) or released into cell cycle. After release, the cells were incubated with or without nocodazole (400 ng/ml). In some experiments SB203580 (10 μM) or DRB (20 μM) was added two hours prior to nocodazole treatment. Cell lysates were then prepared and histone H1 phosphotransferase activities were measured, as equal amount of proteins were loaded and subjected to SDS-PAGE for autoradiography. **B, cdc2 activity is downstream to CK2 and p38 MAP kinase.** Protein extracts from the above treatments were immunoprecipitated with anti-cdc2 antibody, phosphotransferase activity was assayed toward histone H1. Average and standard error of the mean of three separate experiments are shown.

4.2.3. Activation of CK2 and p38 MAP kinase in nocodazole-arrested cells

To determine whether nocodazole-induced activation of p38 and CK2 occurred specifically in M phase cells as a result of spindle assembly defect, or nonspecifically irrespective of the cell cycle, we treated cells with double thymidine block for G1/S synchronization and either collected or released into cell cycle and then subjected to nocodazole (400 ng/ml) for 10 hours. In agreement with previous findings (Takenaka et al., 1998), we also observed that p38 activity in the immune-complex kinase assays (using anti-p38α MAP kinase antibody) in nocodazole-arrested mitotic cells was three-four times higher than that from the metaphase cells (Fig. 11). Furthermore, we have shown that neither the activation nor the phosphorylation of p38 MAP kinase was affected in cells treated with CK2 antisense and exposed to nocodazole (Fig. 11 and 15). We next examined whether CK2 was activated in nocodazole-treated cells or in normal M phase. Immune-complex kinase assays for CK2 revealed that the activity of CK2 from nocodazole-arrested mitotic cell lysates was four times greater than that from interphase cells (control) (Fig. 12). There was negligible stimulation of CK2 activity compared with the control in mitotic cells (shake-off), and the nocodazole-induced activation of CK2 was attenuated by inhibition of p38 MAP kinase using SB203580 (Fig. 12). Collectively, with our studies (Chapter 3) and previous studies (Takenaka et al., 1998), these results raised the possibility that p38 function in the spindle assembly checkpoint is mediated by its down stream target, CK2. Thes result led us to propose that CK2 may function downstream of p38 to maintain the activation of CDK-1 in response to spindle damage.

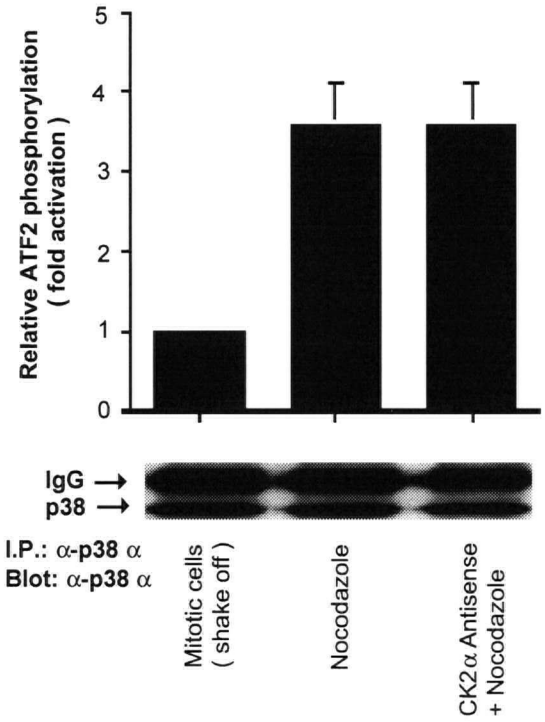

Figure 11: Activation of p38 MAP kinase in nocodazole-arrested cells. p38 protein in cell lysates, prepared from the above treatment (Fig. 10), was immunoprecipitated with anti-p38α antibody and assayed for phosphotransferase activity toward p38 MAP kinase substrate, activating transcription factor 2 (ATF2). The lower panel shows that equal amounts of p38 protein were immunoprecipitated. Average and standard error of the mean of three separate experiments are shown.

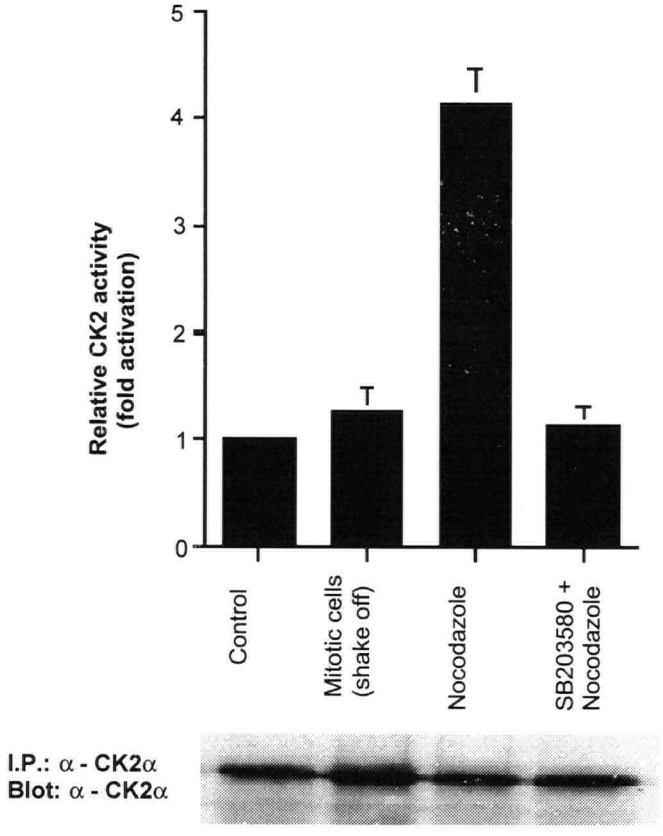

Figure 12: Activation of CK2 in nocodazole-arrested cells is dependent on p38 MAP kinase. CK2 protein in cell lysates, prepared from the above treatments (Fig. 10), was immunoprecipitated with a monoclonal anti-CK2α antibody and assayed for phosphotransferase activity towards the CK2 specific substrate RRADDSDDDDD. Average and standard error of the mean of three separate experiments are shown. The lower panel shows that equal amounts of CK2 protein were immunoprecipitated.

4.2.4. Subcellular immunolocalization of CK2 at different mitotic stages

The centrosome is the major microtubule organizing center in mammalian cells. Functions of the centrosomes are prominently featured during mitosis, where they are required for establishment of spindle bipolarity, spindle microtubule assembly and for determination of the cleavage furrow plane, all of which required for balanced segregation of chromosomes (Winey et al., 1996). Recently, McKendrick et al. (1999) reported increases in p53 and CK2 immunofluorescence within the centrosomes, in C57MG cells treated with the topoisomerase I inhibitor, camptothecin, indicating important functions of these molecules in the microtubule organization. We therefore examined the subcellular localization of CK2 throughout the cell cycle in the presence and absence of the microtubule inhibitor, nocodazole.

We identified centrosomes with a monoclonal antibody to γ-tubulin (anti-γ-tubulin) (Oakley et al., 1990; Joshi, 1994) and the CK2 catalytic subunit by monoclonal antibody to CK2α (anti-CK2α). HeLa cells were synchronized at G0/G1 phase as described in material and methods (Takenaka, et al., 1998) and then released into the cell cycle in the absence and presence of nocodazole. Fig. 13 shows the results of triple label staining with anti-γ-tubulin (fluorescein staining), anti-CK2α (rhodamine) and DNA staining (DAPI). Control experiments were performed by using CK2 α or γ antibody-depleted mouse serum (data not shown). There was no obvious concentration of CK2 in the interphase states (prophase through prometaphase) (Fig. 13), much of CK2 staining remained perinuclear, and diffusely cytoplasmic. During metaphase and anaphase, some CK2 staining was detectable in the spindle (Fig. 13). However, a remarkable increase in

CK2 staining was observed on the spindle body in cells arrested in metaphase by nocodazole treatment. In cells pretreated with SB203580 and then exposed to nocodazole, the staining of CK2 was perinuclear and no detectable staining was observed within the spindle. This result indicates that CK2 may colocalize with the spindle body in response to nocodazole treatment. In addition, it strongly implicates p38 MAP kinase in CK2 translocation to the spindle body in response to spindle damage.

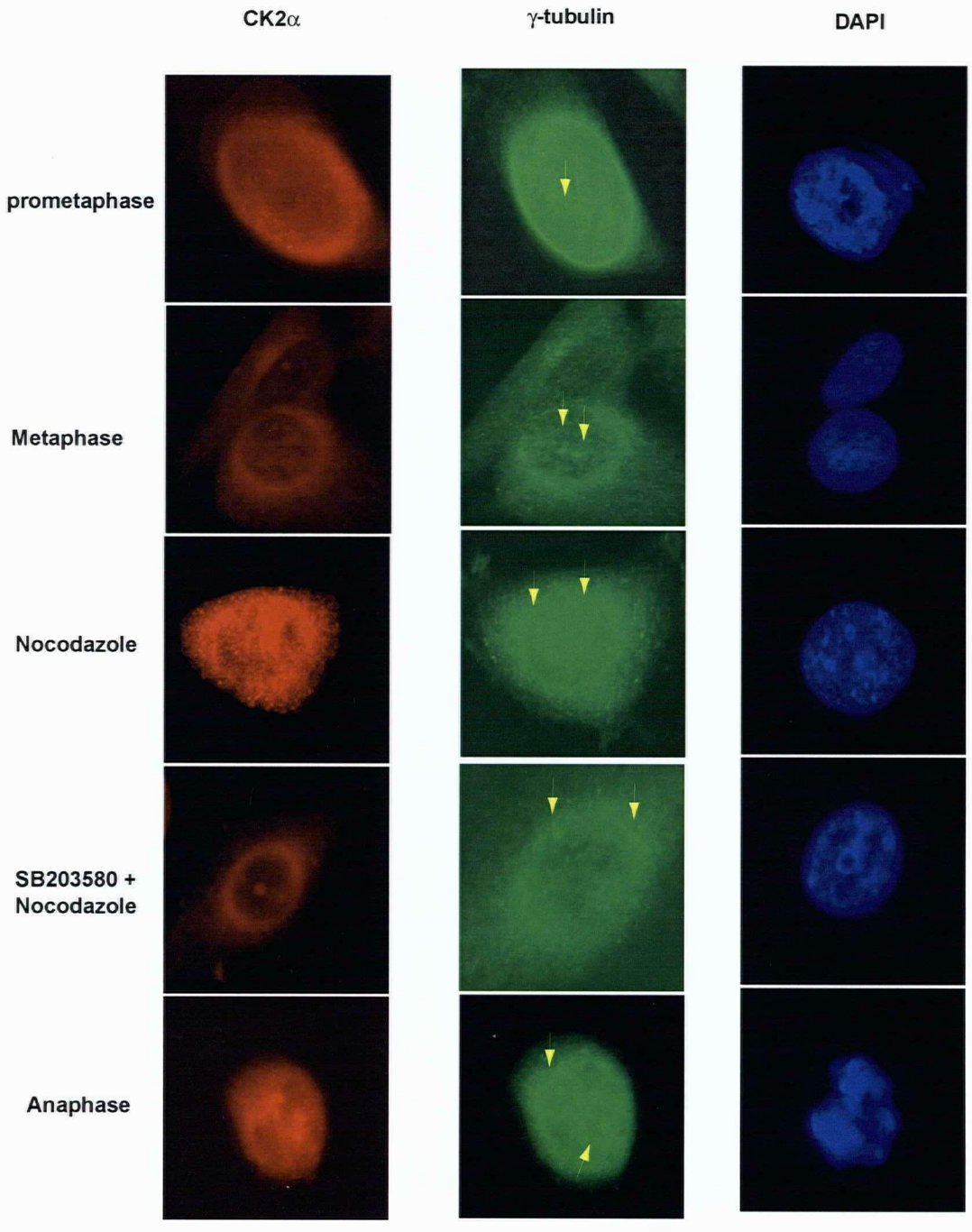

Figure 13: **Subcellular localization of CK2 in HeLa cells.** Exponentially growing HeLa cells were exposed to nocodazole (200ng/ml) for 18 hours in the presence and absence of SB203580, then were fixed and triple-stained with monoclonal antibody to CK2α, antibody to centrosome (γ-tubulin) and DAPI. Arrows indicate the centrosome localization. Cells observed with a x 100 oil immersion objective lens.

4.3. Discussion

As I discussed in the Introduction, the cell cycle G2/M transition and progression are driven by the activation of CDK-1 (cdc2-cyclin B) complex. This process is a critical event to ensure that chromosomes attach to and become aligned on the mitotic spindle before the inactivation of the CDK-1. Thus exit from mitosis and progression to anaphase requires the subsequent inactivation of CDK-1. This process of cell division is highly monitored by a checkpoint termed the spindle assembly checkpoint. Inhibition of spindle assembly with depolymerizing drugs such as nocodazole, activates the spindle assembly checkpoint and prevents the onset of anaphase. This event involves mitotic arrest by elevation of CDK-1 and inhibition of its degradation.

Previous work by Takenaka et al. (1998) has shown that p38 MAP kinase is a component of the spindle assembly checkpoint. It has been shown that activation of the spindle assembly checkpoint by disruption of the spindle with nocodazole results in activation of p38 MAP kinase and mitotic arrest (Takenaka et al., 1998). During mitotic arrest by nocodazole, p38 MAP kinase was shown to maintain activation of CDK-1 in response to spindle damage, this is to ensure that cells cannot override the mitotic arrest, induced by the microtubule inhibitor (Takenaka et al., 1998).

Here we showed that the function of p38 MAP kinase in the spindle assembly checkpoint activation might due to the activation of its down stream target CK2. Our results have shown that depletion of CK2 by CK2 antisense resulted in failure of the mitotic arrest by nocodazole (Fig. 8 to 9). This is consistent with the results obtained by using dominant negative p38 MAP kinase and p38 specific inhibitor, SB203580

(Takenaka et al., 1998). We have shown that the number of mitotic cells induced by nocodazole were significantly reduced by CK2 depletion. To investigate whether CK2 depletion causes the loss of the checkpoint function, we synchronized CK2-depleted HeLa cells at G1/S phase by double thymidine block and then released them into cell cycle. Before mitosis, we treated the cells with nocodazole. Our results show that the failure of the mitotic arrest correlated with inhibition of cdc2 activity (Fig. 10). Similar results were obtained using the CK2 inhibitor DRB (Fig. 10). Our findings revealed that CK2 was activated only in response to nocodazole and was not activated during normal mitosis (Fig. 12). Therefore, this result clearly shows that CK2 is not a component of the spindle assembly but it is a component of the spindle checkpoint. Since CK2 depletion or inhibition compromised the spindle checkpoint activation by nocodazole, we determined whether p38 MAP kinase is part of the same pathway by using the specific inhibitor of p38, SB203580. Similar results were observed. The activation of CDK-1 by nocodazole was suppressed in cells treated with SB203580 (Fig. 10). The activation of CK2, in response to nocodazole, was suppressed by the p38 inhibitor (Fig. 12). However, the activation of p38 MAP kinase was not affected by CK2 antisense (Fig. 11). Therefore we conclude from these results that CK2 functions as a component of the spindle assembly checkpoint in the somatic cell cycle in response to spindle damage, perhaps by sustaining or maintaining the activity of CDK-1 and mitotic arrest. This function is likely mediated by the CK2 upstream target, p38 MAP kinase.

Finally, we observed a striking immunofluorescent staining of the CK2α within the spindle body in response to nocodazole (Fig. 13). This significant staining of CK2 in nocodazole-treated cells was dramatically reduced and became perinuclear in cells

pretreated with SB203580. Interestingly, our results also show that the CK2 staining within the spindle body during interphase and metaphase was negligible (Fig. 13). Our data strongly indicate a translocation of CK2 to the spindle body in response to spindle damage. The translocation of CK2 implicates p38 MAP kinase signaling pathway, indicating an cooperation between these two molecules following spindle damage.

5. PROTEIN KINASE CK2 IS REQUIRED FOR p53 FUNCTION IN RESPONSE TO SPINDLE DAMAGE

5.1. Rationale and Hypothesis

Protein kinase CK2 phosphorylates the human tumor suppressor p53 at Ser-392 (Ser-386 in murine p53) (Meek et al., 1990; Herrmann et al., 1991). Phosphorylation by CK2 has been shown to enhance p53 sequence-specific DNA binding *in vitro* (Hupp et al., 1992), which in turn contributes to its transactivation function *in vitro* (Mundt et al., 1997). *In vivo*, the phosphorylation of p53 protein at the CK2 site has been reported to regulate p53-specific transcriptional repression (Hall et al., 1996). Recently, the tumor suppressor p53 has been proposed to be a component of the spindle assembly checkpoint, indicating its essential role to ensure maintenance of diploidy and DNA integrity (Cross et al., 1995; Lanni and Jacks, 1998).

Our latter results indicated a role for CK2 in response to spindle damage. We further investigated whether CK2 function in spindle assembly checkpoint involves the p53 molecule.

5.2. Results

5.2.1. Nocodazole-induced p53 Ser- 392 phosphorylation is mediated by CK2 and is SB203580 sensitive

To test the hypothesis that the p53 is a downstream target of the stress stimuli anisomycin, arsenite, and TNFα, we performed immunoblotting studies of lysates from HeLa cells, that had been treated with these factors, using a p53 Ser-392 phosphorylation site-specific antibody. As shown in Fig. 14 the treatment of serum-deprived HeLa cells with any of these agents enhanced the phosphorylation of p53 at this site. The phosphorylation of p53 in response to anisomycin and TNFα, but not arsenite, was markedly attenuated by either DRB or SB203580. This result indicates that the phosphorylation of p53 at Ser-392 site in response to anisomycin or TNFα was mediated by p38 MAP kinase and CK2. In the case of arsenite, we have observed no change in p53 phosphorylation in cells pretreated with the p38 MAP kinase inhibitor, SB203580 (Fig. 14). Thus, we suggest that there may be an alternative pathway, in addition to the p38 MAP kinase-CK2 route, by which p53 can be phosphorylated at the Ser-392 site. Interestingly, it has been shown that arsenite induces p53 accumulation and phosphorylation through an ATM-dependent pathway (Yih and Lee, 2000).

We reexamined the above finding in the context of mitotic arrest induced by nocodazole. We synchronized HeLa cells transfected with CK2 sense, antisense oligonucleotides, or buffer at G1/S phase by double thymidine block and then released them into cell cycle in the presence and absence of nocodazole. Figure 15 shows that the

hyperphosphorylation of p53 at Ser-392 due to nocodazole, was completely suppressed in cells either transfected with AS or treated with the p38 specific inhibitor SB203580. This effect was independent of changes in p53 protein expression (lower panel). The depletion of CK2 did not affect the phosphorylation or the activation of p38 upon nocodazole treatment (Fig. 11 and 15), confirming its role downstream of p38 MAP kinase activation. Our results also show that p53 phosphorylation at Ser-392 in normal mitotic cells was negligible. Thus, these results indicate that CK2 phosphorylates p53 at Ser-392, due to the nocodazole-induced activation of the spindle assembly checkpoint, and p38 MAP kinase is an upstream regulator. The phosphorylation of p53 at Ser-392 in response to the mitotic arrest by nocodazole correlates nicely with the activation of CK2 and p38 MAP kinase in the event of mitotic arrest by nocodazole (Fig. 11 and 12).

It should be noted that HeLa cells are known to harbor human papilloma virus (HPV), which has been shown to target p53 for degradation (Scheffner, 1993). However, it has been reported that cervical carcinoma cells, (including HeLa), express functional wild-type p53, which is sufficient to activate the transcription of cellular target genes (Butz et al., 1995). We were able to detect p53 protein in these cells and p21 was induced (data not shown). Fig. 15 shows the level of p53 expression observed in our studies.

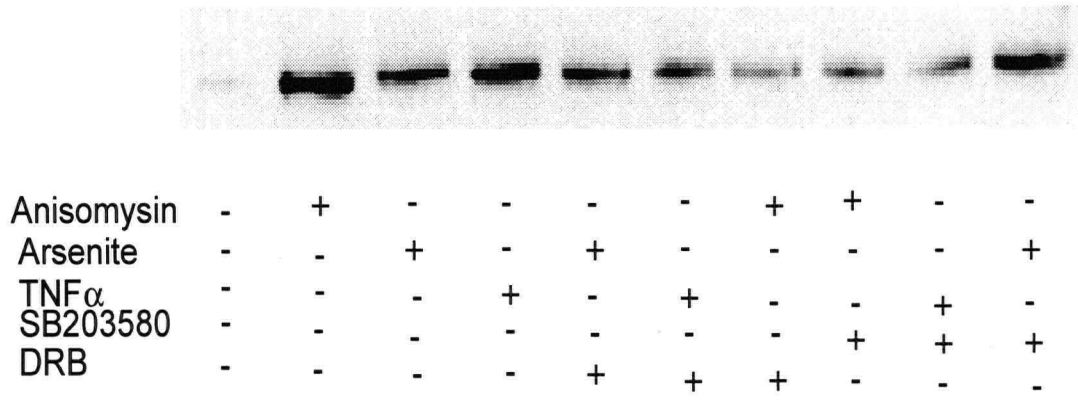

Figure 14: Stress factor-induced phosphorylation of p53 at Ser-392 is dependent on CK2 and p38 MAP kinase. HeLa cells were serum starved (0.5%) for 18 hours and then treated with anisomycin (10 mg/ml for 30 min), arsenite (50 μM for 15 min), or TNFα (20 ng/ml for 15 min). Cell Lysates were immunoblotted with anti- p53 Ser-392. Figure shows enhanced phosphorylation of p53 as detected with a p53 Ser-392 phospho-site antibody. The increased phosphorylation in response to anisomycin and TNFα could be reduced by pretreatment of the cells with the CK2 inhibitor DRB (20 μM) and by the p38 MAP kinase inhibitor SB203580 (10 μM). Results are representative of three experiments.

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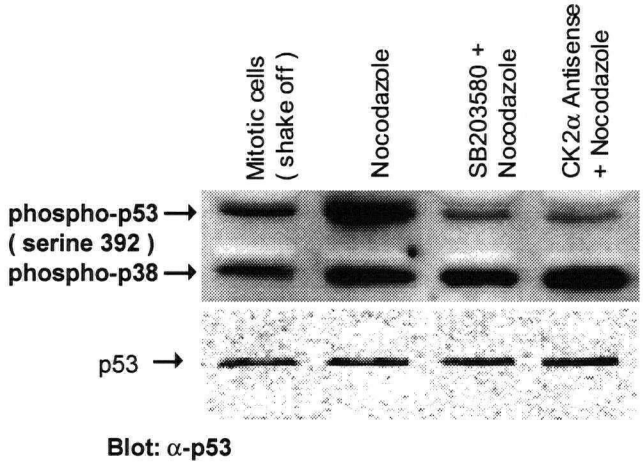

Figure 15: CK2 is the major kinase phosphorylating p53 at Ser-392. HeLa cells were synchronized at G1-S by double thymidine block and then released into cell cycle. Mitotic cells were either collected (shake off) after 9 hours or were incubated with or without nocodazole (400 ng/ml) for 10 hours. In some experiments SB203580 (10 μM) or DRB (20 μM) was added two hours prior to nocodazole treatment. Cell lysates from the above treatment were immunoblotted with phospho site-specific antibodies to p53 (Ser-392) and p38α, or polyclonal antibody to p53 protein.

5.2.2. Depletion of CK2 causes an override of mitotic arrest following spindle damage

Having demonstrated an effect of CK2 depletion on mitotic arrest (Fig. 8 to 9), CDK-1 activity and p53 phosphorylation (Fig. 10 and Fig 15), we wanted to determine the cell cycle characteristics of these cells in more detail. Therefore, exponentially growing HeLa cells were transfected with CK2 AS, S or buffer alone. Cells were then synchronized in G0/G1 phase by serum starvation (0.5%) for 30 hours and then released into the cell cycle by addition of 10% serum containing medium, as previously described (Takenaka et al., 1998). Nocodazole (200 ng/ml) was added before the cells entered mitosis for the indicated times shown. Fig. 16 shows the FACS analysis data of synchronized HeLa cells, in the presence and absence of nocodazole. Fig. 16 shows that cells, after 6 hours following release into the cell cycle, progressed through S phase and entered G2 and mitosis with similar kinetics (Fig. 16). However, from 12 hours, the behavior of these populations was dramatically different. Sense-treated cells exposed to nocodazole continued to accumulate in mitosis for the next 12 hours with DNA content of 4N (Fig. 16). The time-dependent increase of cdc2 activity (Fig. 17), as well as corresponding cyclin B expression (Fig. 18A), and mitotic index (Fig. 20) by 18 hours, all confirm that cells remained blocked in mitosis upon exposure to nocodazole. Significantly, cells depleted of CK2 appeared to progress into the cell cycle, compromising the mitotic block (Fig. 16), with a large population of cells progressing into a G1-like state with DNA content of 2N populations by 24 hours. The mitotic index (Fig. 20) of the cycling cells indicates that ≥30% of cells entered mitosis by 24 hours, despite disruption of mitotic assembly. Thus, CK2-depleted cells treated with

nocodazole appeared to exhibit an override of the mitotic assembly checkpoint and an apparent reentry into S phase. This was also manifested by an alteration of cyclin B expression (Fig. 18A) and cdc2 inactivation (Fig. 17).

To further investigate if the expression of cyclin B correlated with phosphorylation-dependent inactivation of cdc2, the electrophoretic mobility of cdc2 was used to assess whether it was phosphorylated on Thr-14, Tyr-15 or both. Figure 18B shows the phosphorylation stages of cdc2 upon nocodazole stimulation in the presence and absence of CK2 antisense. The most gel-retarded form of cdc2, species 'a', is phosphorylated on both Thr-14 and Tyr-15 whereas the intermediate form, species 'b', is phosphorylated on Thr-14 or Tyr-15, but not both (Atherton-Fessler et al., 1994; Liu et al., 1997). The fastest migrating form of cdc2 (species 'c') is not phosphorylated on either Thr-14 or Tyr-15, and represents either the active form of the kinase (phosphorylated on Thr-161 and bound to cyclin B), or cdc2 that is not bound to cyclin (monomeric, inactive cdc2) (Atherton-Fessler et al., 1994; Liu et al., 1997). Figure 18B demonstrates that the phosphorylation state of cdc2 remains unchanged in AS-transfected cells, whereas cyclin B turnover is altered (Fig. 18A). Collectively, these results indicate that the inhibition of cdc2 activation in CK2-depleted cells upon nocodazole exposure, was not correlated with changes in the phosphorylation of cdc2, but was likely due to the altered cyclin B kinetics. Similar results were also obtained by using the CK2 inhibitor, DRB (data not shown).

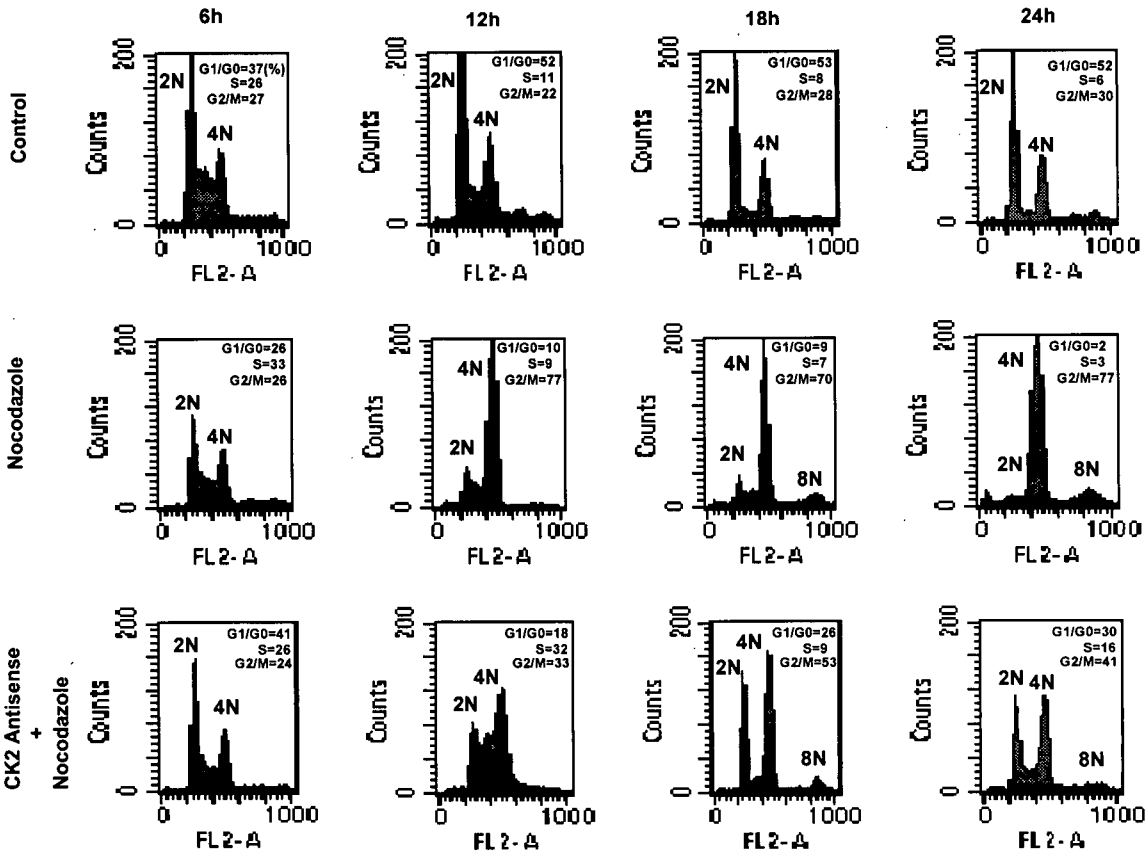

Figure 16: Mitotic arrest is compromised in the absence of CK2. Synchronized HeLa cells at G0/G1, by serum starvation (0.5%) for 30 hours were treated with CK2 Sense (control) or CK2 Antisense and then released into cell cycle by addition of 10% serum. Cells were collected for flow cytometric analysis in the presence and absence of nocodazole (200 ng/ml) at the indicated time. The percentage of the DNA contents is shown within the figure.

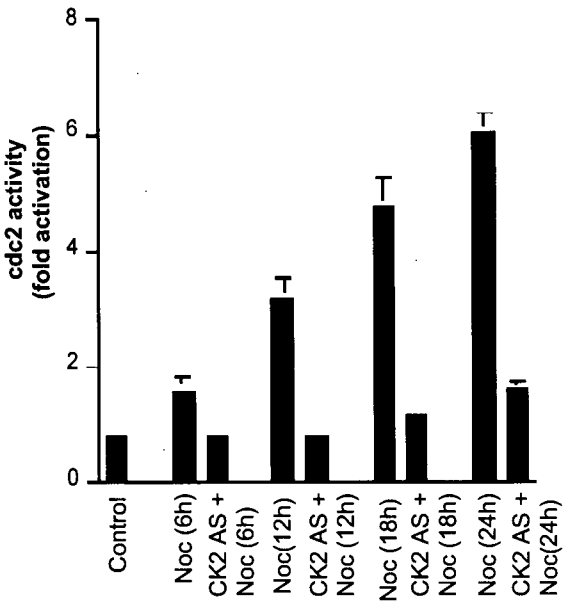

Figure 17: Inhibition of cdc2 activity in CK2-depleted HeLa cells. Transfected cells with CK2 Sense and CK2 Antisense oligonucleotides were exposed to nocodazole (200 ng/ml) for the indicated times or left untreated. Control is a representative of cells transfected with CK2 Sense or left untreated. Cell lysates were immunoprecipitated with anti-cdc2 antibody and assayed for cdc2 phosphotransferase activity toward histone H1. Average and standard error of the mean of three separate experiments are shown.

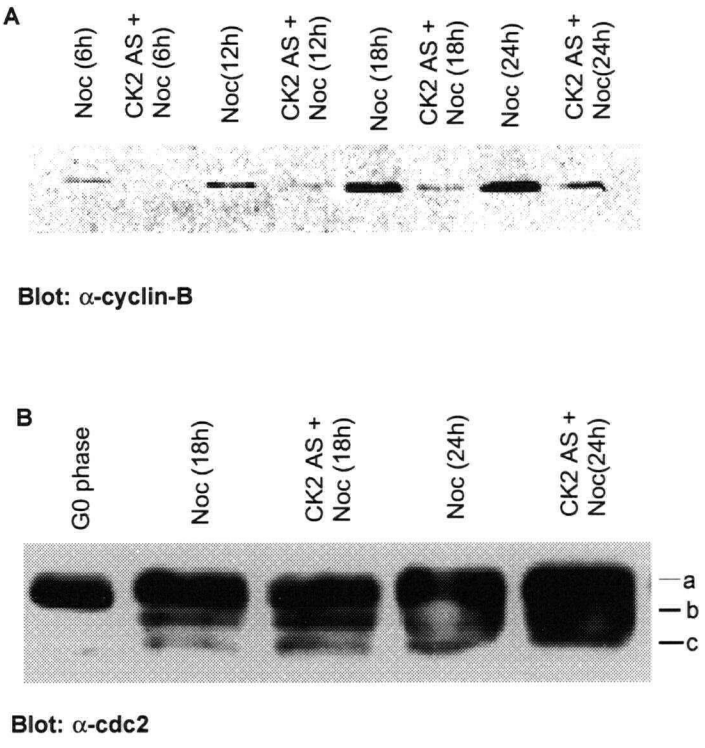

Figure 18: Phosphorylation of cdc2 is independent of cyclin B degradation in CK2-depleted cells. Transfected cells with CK2 Sense and CK2 Antisense oligonucleotides were synchronized at G0/G1 phase and then exposed to nocodazole (200 ng/ml) or collected at G0/G1 phase. Lysates from above treatment were either subjected to immunoblotting with anti-cyclin B **(A)** or cdc2 **(B)** protein, (a), (b) and (c) indicate the different stages of the electrophoretic mobility of cdc2 protein.

5.2.3. Mitotic spindle checkpoint compromise requires p53 protein

p53 has been shown to be involved in G2/M phase to maintain cell diploidy (Ko, 1996). Several studies have shown that $p53^{-/-}$ cells accumulate 4N, 8N and 16N populations upon exposure to microtubule inhibitors for >48 hours (Cross et al., 1995; Lanni and Jacks, 1998). From our findings thus far, we were unable to directly implicate p53 in the altered spindle checkpoint arrest observed following CK2 depletion. Therefore, we investigated this hypothesis in the colorectal MIN (microsatellite instability) HCT116 human colon cancer cell line (Bunz et al., 1998), because it has an intact spindle-dependent checkpoint (Bunz et al., 1998; Cahill et al., 1998). As expected, HCT116 cells with either wild type (wt) $p53^{+/+}$ or with disrupted $p53^{-/-}$ alleles (Bunz et al., 1998) [we have confirmed that there was no detectable p53 protein in $p53^{-/-}$ cell line, (data not shown)], were shown to be arrested in M phase upon nocodazole exposure (Fig. 19). However, $p53^{+/+}$ cells transfected with CK2 AS, in common with HeLa cells similarly treated, also appeared to show a dramatically compromised mitotic spindle checkpoint with 32% of the cells in G0/G1 compared with only 3% for $p53^{-/-}$ cells, after 24 hours (Flow cytometric statistical analysis of Fig. 19). Furthermore approximately 30% of the cells had reached mitosis by 24 hours. In striking contrast, $p53^{-/-}$ cells transfected with CK2 AS maintained a high mitotic index comparable with nocodazole treated $p53^{-/-}$ cells (Fig. 20), accumulating 4N with diminished 2N populations (Fig. 19). Thus, it appeared that the normal checkpoint status of MIN cells, HCT116 with wild type p53, following CK2 depletion and spindle damage, is converted to that characteristic of CIN (chromosomal instability) cells (Cahill et al., 1998).

We evaluated whether cyclin B expression was affected in $p53^{-/-}$ and $p53^{+/+}$ cells. Figure 21 shows that cyclin B was not affected by depletion of CK2 in $p53^{-/-}$, while the $p53^{+/+}$ exhibited a down-regulation of cyclin B expression in response to antisense CK2 depletion (Fig. 21) in keeping with the HeLa cell data. Another important finding to emerge from investigation of the HCT116 ($p53^{-/-}$ or $p53^{+/+}$) cells was that antisense depletion of CK2 did not influence the ability of the cells to reach the mitotic checkpoint. This is exemplified by the fact that both at 18 hours and at 24 hours, there are equivalent fractions of cells at the G2/M stage for both nocodazole, and nocodazole + antisense treated $p53^{-/-}$ cells (60% and 61% at 18 h, and 67% and 65% at 24 hours, respectively). In addition, the FACS analysis profile of CK2 AS-treated HCT116 $p53^{+/+}$ cells alone (Fig. 19) shows that these cells exhibit the same kinetics as cells transfected with CK2 sense (Fig. 19) or left untreated (data not shown). These results reduce the possibility that AS treated HeLa cells underwent an S phase delay. Furthermore, our results showed that neither the activity nor the protein levels of CK2 were affected in HCT116 $p53^{-/-}$ cells (results not shown). Therefore, these studies seem to indicate that abrogation of the mitotic assembly checkpoint and cyclin B degradation are dependent on p53 protein, and in addition, are regulated by CK2.

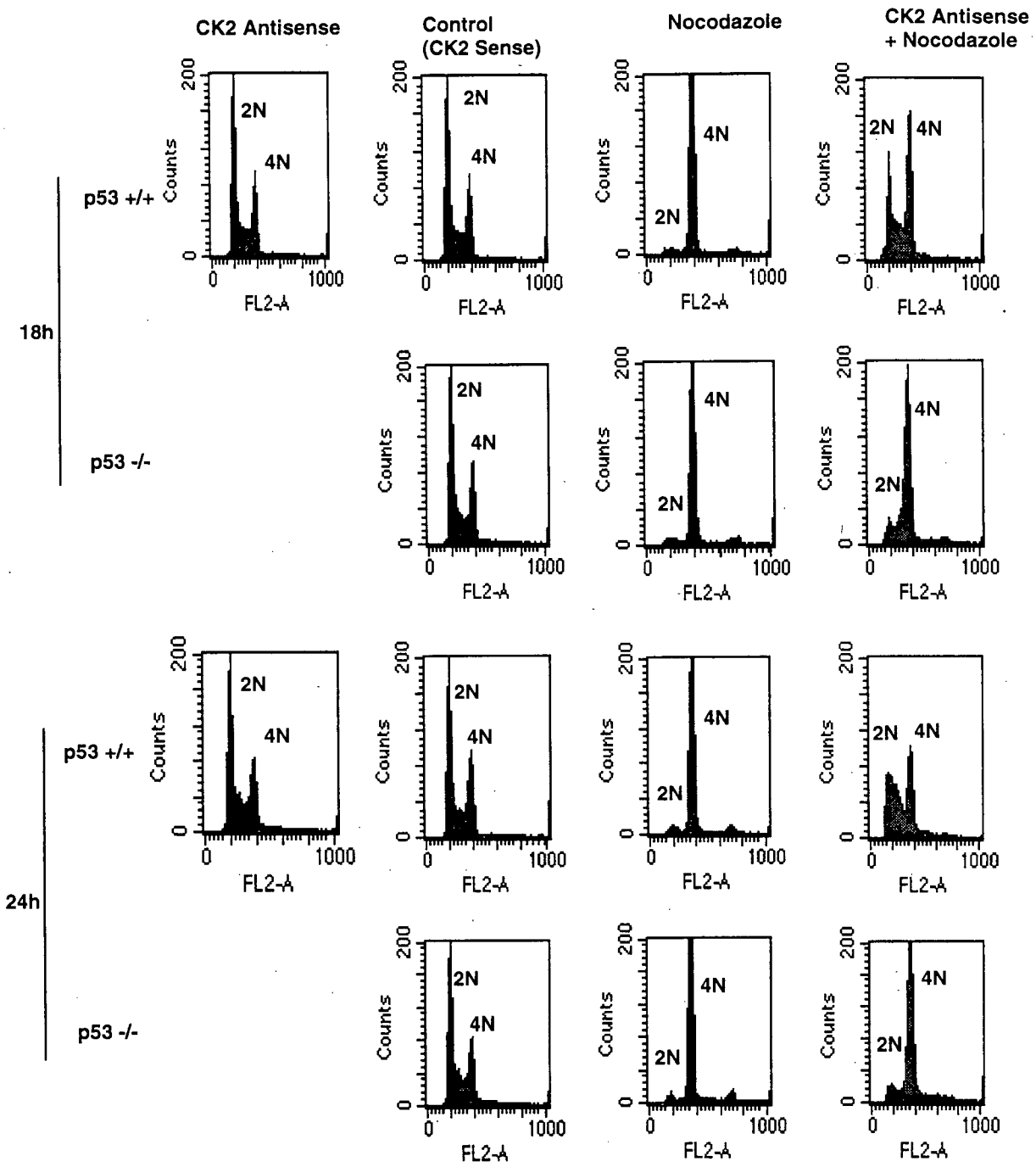

Figure 19: p53 is required for CK2 to override the mitotic arrest. Synchronized HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cell line at G0/G1, by serum starvation (0.5%) for 30 hours were treated with CK2 Sense (control) or CK2 Antisense and then released into cell cycle by addition of 10% serum. Cells were collected for flow cytometric analysis in the presence and absence of nocodazole (200 ng/ml) at the indicated time.

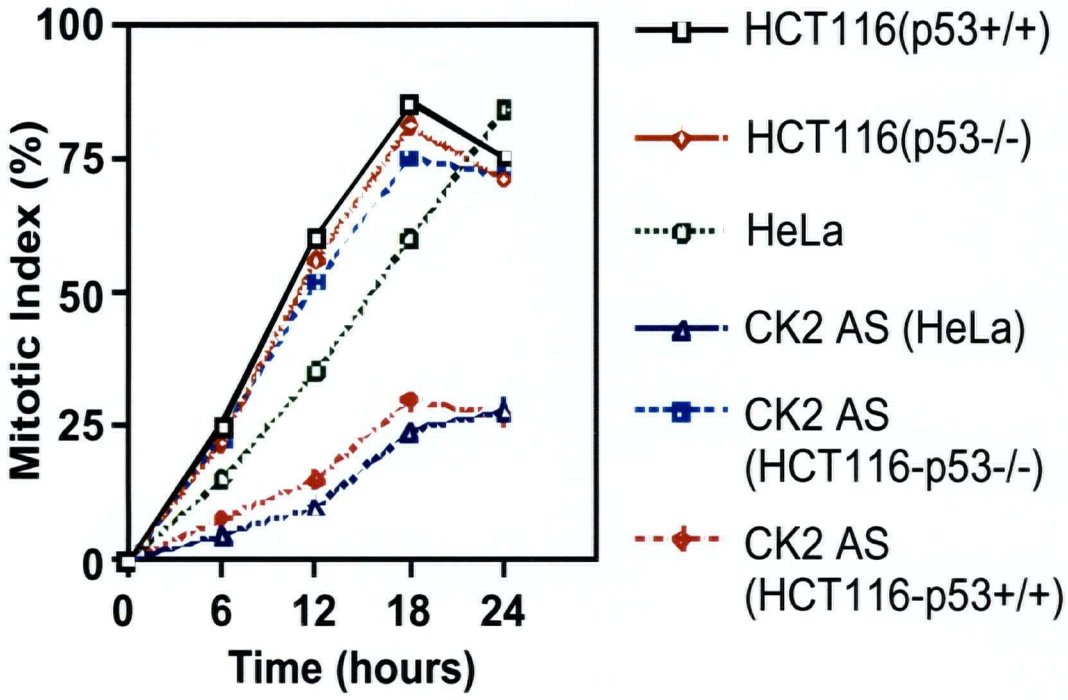

Figure 20: Mitotic indices of HCT116 and HeLa cells. Cells were treated with nocodazole (200 ng/ml) at the indicated times. Nuclei with condensed, evenly stained chromosomes with Hoescht 33258 were scored as mitotic cells. At least 600 cells were visualized in three independent experiments.

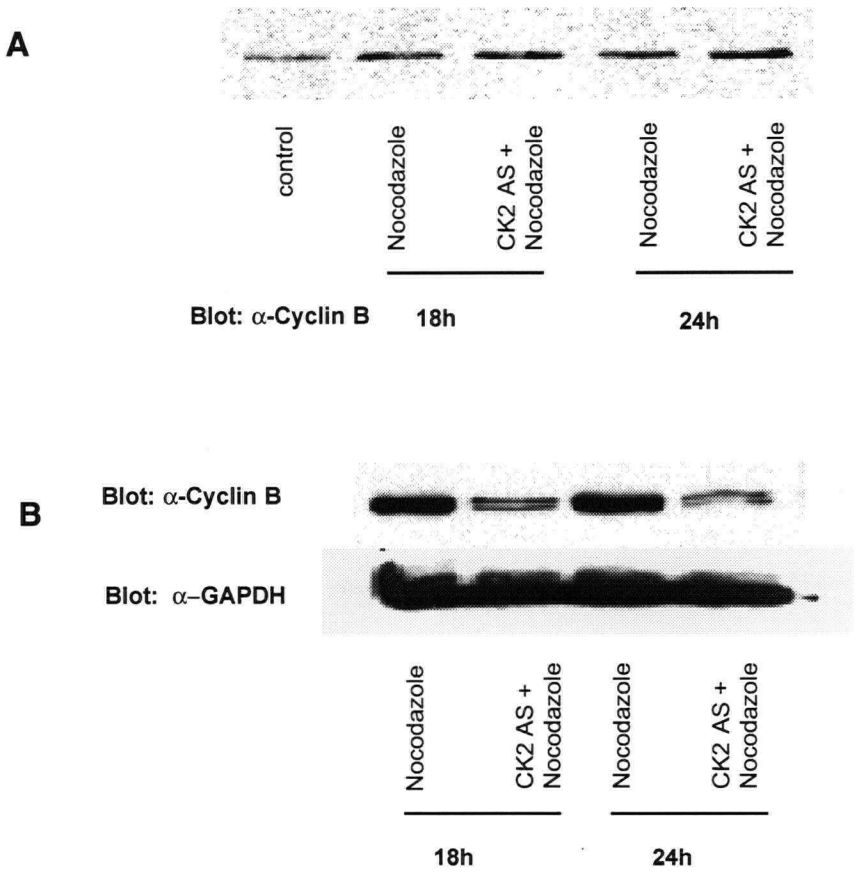

Figure 21: Cyclin B expression in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells. CK2-depleted (AS-treated) and Sense-treated $p53^{-/-}$ (**A**) and $p53^{+/+}$ (**B**) cells were exposed to nocodazole (200 ng/ml) at the indicated times, or left untreated (control). Lysates were immunoblotted with anti-cyclin B. Equal amount of proteins is shown in the lower panel by immunoblotting with ant-GAPDH polyclonal antibody (B).

5.2.4. Cells transfected with CK2 antisense escaped nocodazole-induced cell death

The tumor suppressor p53 has been shown to mediate apoptosis in several cell types (Moll and Zaika, 2001). Loss of p53 prevents thymocytes from undergoing apoptosis after gamma irradiation, and decreases the efficacy of chemotherapy *in vitro*, and *in vivo* (Lowe et al., 1993; Ko and Prives, 1996; Moll and Zaika, 2001). To examine cell death in cells transfected with CK2 AS, we analyzed and quantified the phenotypic changes characteristic of apoptotic cells by double staining HeLa cells with annexin V and propidium iodide (PI). Exponentially growing control or AS-transfected cells were exposed to nocodazole, and then harvested every 6 hours. Figure 22 shows that 22% of control cells had undergone apoptosis after 24 hours of exposure with nocodazole. These observations are consistent with previous studies in certain cell lines, which undergo apoptosis following persistent exposure to nocodazole (Minn et al., 1996; Taylor and McKeon, 1997). In contrast, cells transfected with CK2 AS showed a significant, reproducible 50% reduction in apoptosis. An even greater reduction in apoptosis (72%) consequent upon CK2 depletion was observed using HCT116 $p53^{+/+}$ cells (Fig. 23A). As expected, $p53^{-/-}$ cells exhibited a much lower frequency of apoptosis in response to nocodazole in comparison to $p53^{+/+}$ cells (10% vs. 32.2%). Furthermore, our results show that there is no change in the percentage of apoptotic cell fractions in HCT116 $p53^{-/-}$ cells transfected with CK2 AS and exposed to nocodazole (Fig. 23B). In further support the co-operativity between CK2 and p53, it is apparent from this data that $p53^{-/-}$ cells do not exhibit a reduction of apoptotic cell fraction upon CK2 depletion.

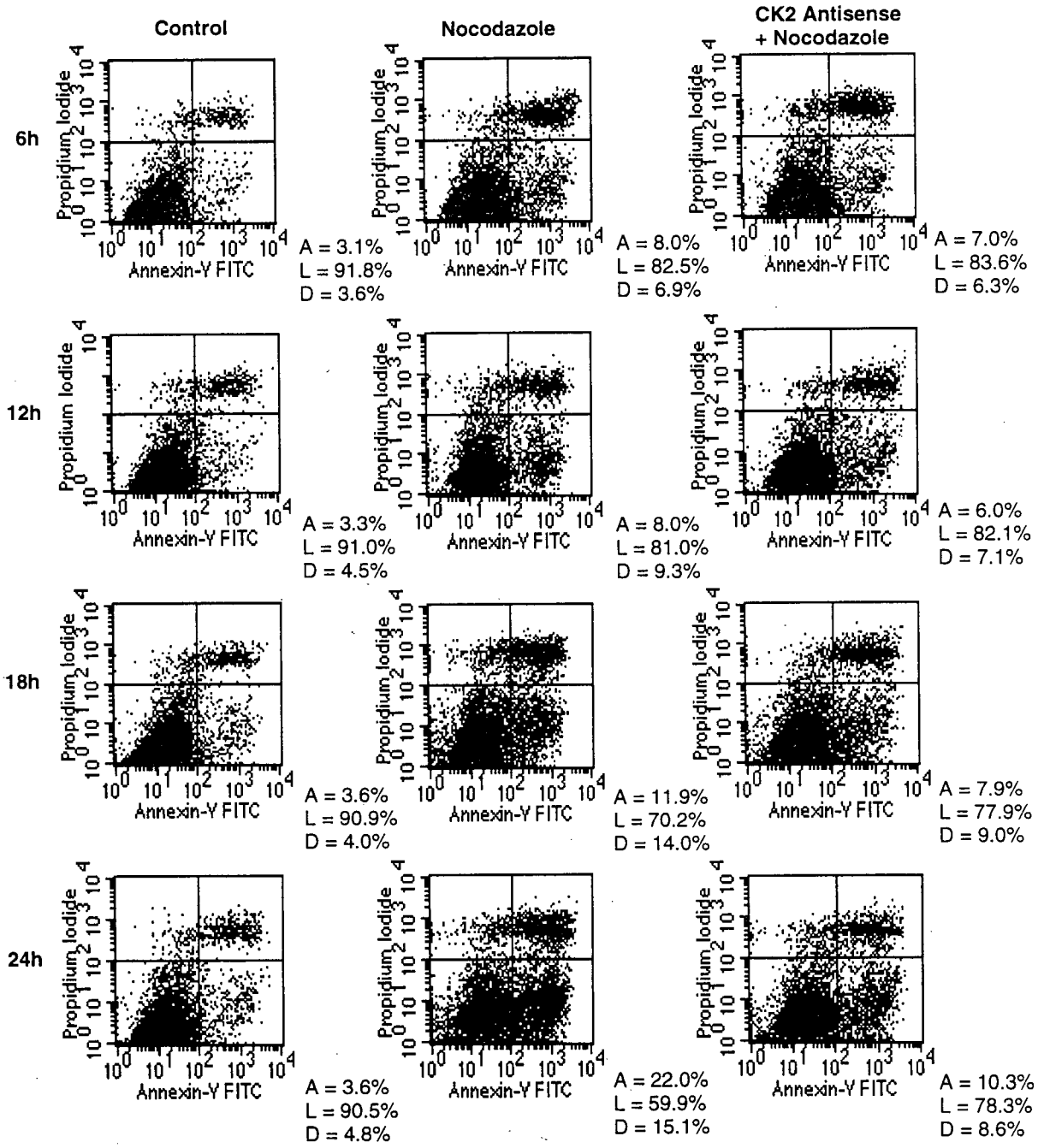

Figure 22: Suppression of apoptosis in the absence of CK2 in HeLa cells. Quantitative analysis of the apoptotic cells using annexin-V FITC in exponentially growing HeLa cells-transfected with CK2 antisense or Sense-treated (control) in the presence and absence of nocodazole (100 ng/ml) for 24 h. The flow cytometric analysis was performed on 100,000 cells and the percentage of apoptotic (A, the right lower quadrant), live (L, the left lower quadrant) and dead (D, the top right quadrant) cells was measured in three separate experiments.

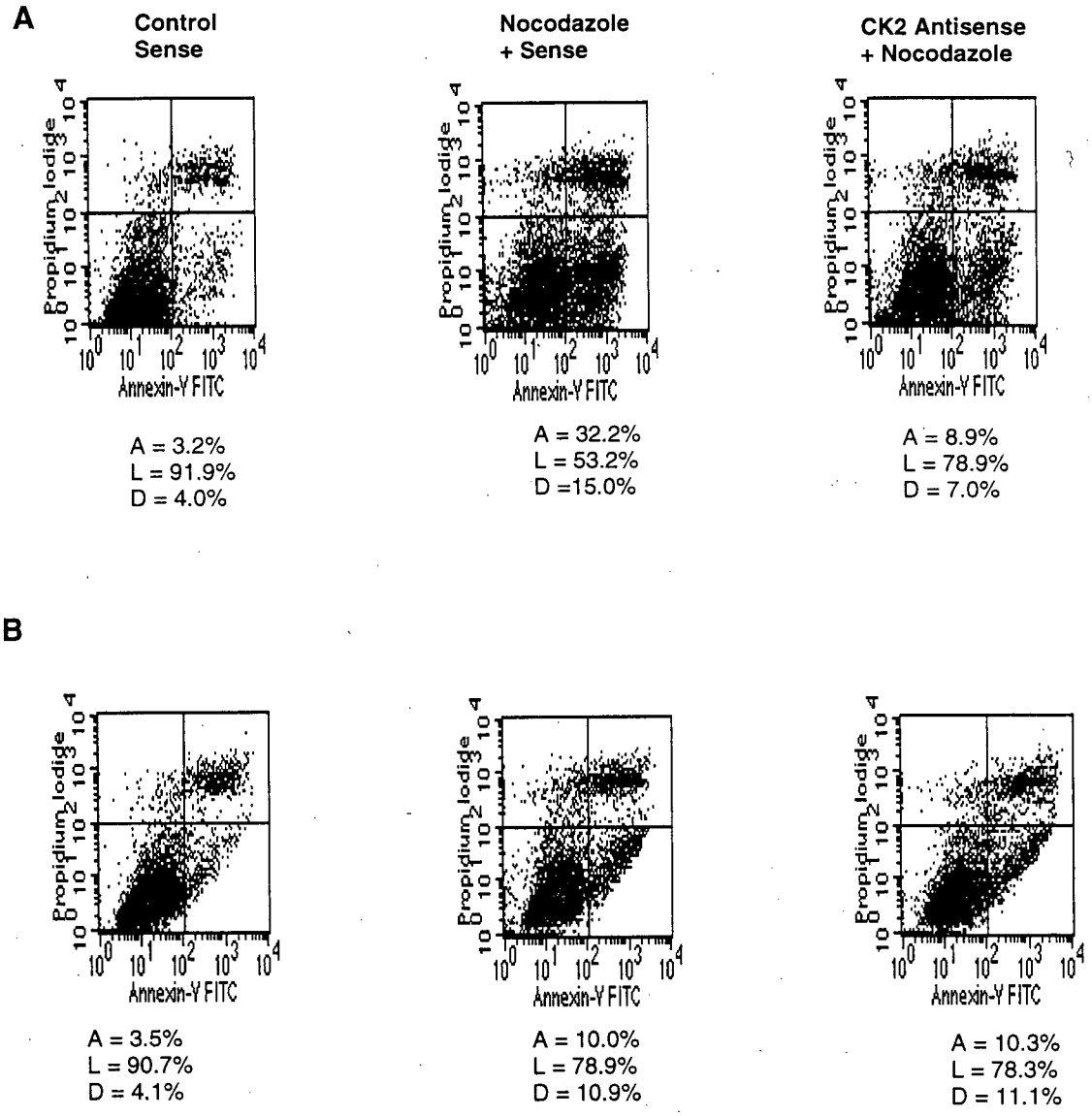

Figure 23: Suppression of apoptosis in the absence of CK2 in HCT116 cells. Quantitative analysis of the apoptotic cells using annexin-V FITC in exponentially growing HCT116 $p53^{+/+}$ **(A)** and HCT116 $p53^{-/-}$ **(B)**, transfected with CK2 Antisense or Sense-treated (control) in the presence and absence of nocodazole (100 ng/ml) for 24 h. The flow cytometric analysis was performed on 100,000 cells and the percentage of apoptotic (A), live (L) and dead (D) cells was measured in three separate experiments.

5.2.5. Abnormal centrosome amplification consequent upon CK2 depletion

Loss of regulation of centrosome duplication cycle, or uncoupling of the centrosome duplication cycle from the DNA replication cycle leads to abnormal amplification of centrosomes, which in turn profoundly affects mitotic fidelity. Centrosome defects have been implicated in mitotic abnormalities and tumor progression in human cancer (Carroll et al., 1999; Lingle et al., 1999). Both initiation and centrosome duplication and suppression of reduplication appears to be controlled by p53 gene (Fukasawa et al., 1996). To investigate whether the loss of CK2 has a similar effect on centrosome behavior, we synchronized HeLa cells transfected with CK2 AS or Sense at G0/G1 phase, then released them into the cell cycle. Nocodazole was then added before the entry into M phase and cells were harvested and fixed every one hour. Cells were stained with anti-γ tubulin for centrosome visualization and DAPI for nuclei. Figure 24 shows that CK2-depleted cells had initiated anaphase despite spindle damage, dividing into two daughter cells with abnormal positioning of the two centrosomes (A). Some cells had formed multiple copies of centrosomes (B) which indicated that they had undergone reduplication. This result shows that the acquisition of an abnormal number of centrosomes was due to direct consequence of CK2 depletion.

Surprisingly, we also found that these cells had undergone cytokinesis despite the abnormal centrosome amplification, producing cells with two copies or more of centrosomes (Fig. 24C). Figure 24D shows that the Sense-transfected HeLa cells, exposed to nocodazole, arrest in mitosis. Fig. 24E-F shows normal mitotic spindle assembly and anaphase in CK2-depleted cells, in the absence of nocodazole. These cells

show normal spindle with a typical bipolar array of anti-parallel microtubules organized by two centrosomes at the poles (mitosis) (E), and anaphase with typical alignment of centrosomes in the two daughter cells (F). Thus, these observations may implicate CK2 in the regulation of centrosome duplication.

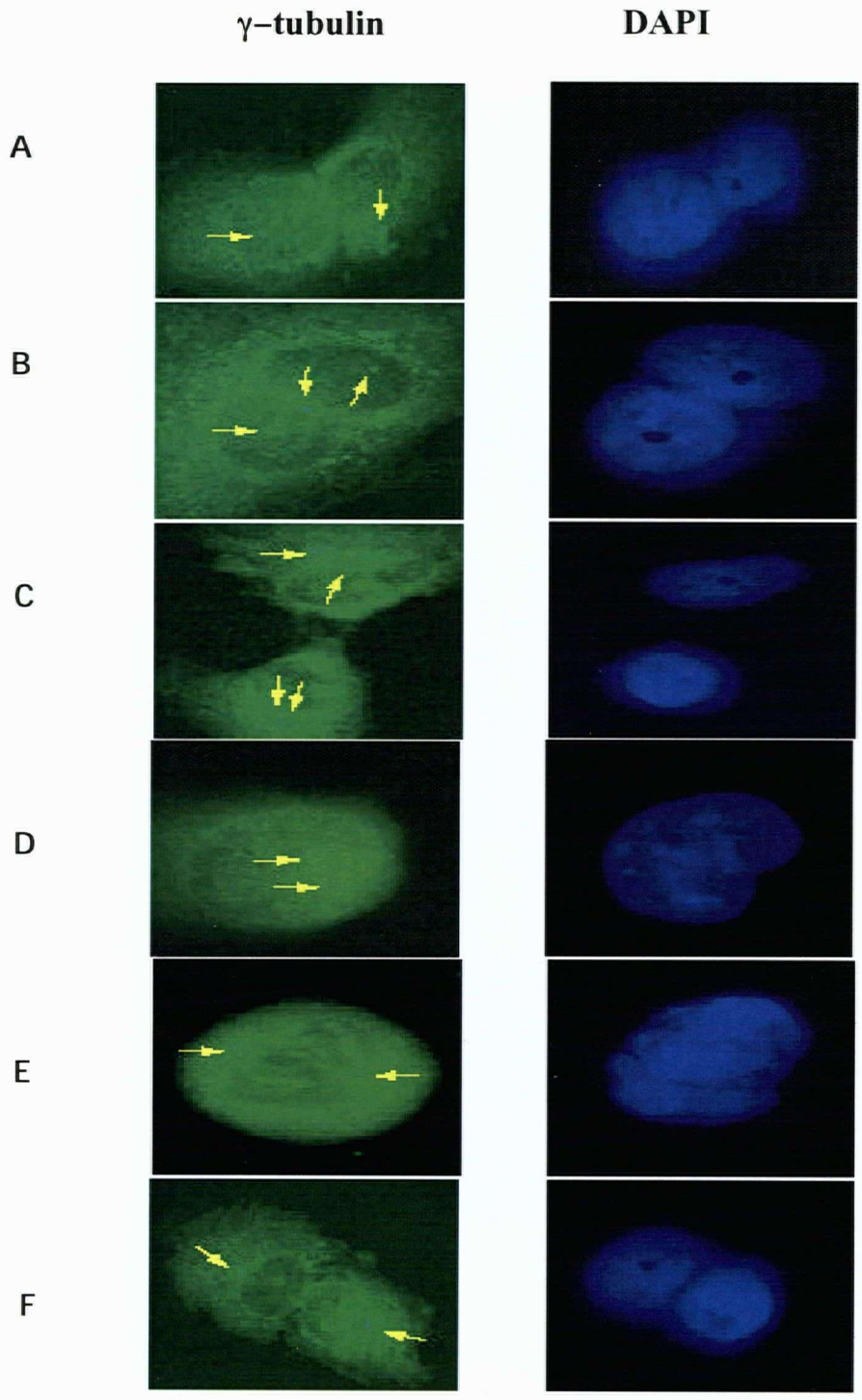

Figure 24: Abnormal amplification of centrosomes following AS- mediated CK2 depletion. Synchronized HeLa cells transfected with CK2 antisense and sense oligonucleotides were immunostained with anti-γ tubulin, DAPI and CK2α antibodies. **A** to **C,** CK2-depleted cells were exposed to nocodazole (200ng/ml),undergoes anaphase **(A**to **B)** and Cytokinesis **(C)**. Arrows indicate the centrosome localization. **D,** nocodazole-arrested CK2 sense-transfected cells; **E,** CK2-depleted cells in early anaphase and **F,** control cells in late anaphase (in the absence of nocodazole). Cells observed with a 100 x oil immersion objective lens.

5.3. Discussion

In this study we have demonstrated for the first time, a significant functional co-operation between protein kinase CK2 and p53, in modulation of cdc2 phosphotransferase activity and the mitotic checkpoint arrest following nocodazole-induced spindle damage. This effect was influenced by the p38 MAP kinase, as both the biochemical events and the cell-cycle changes could be reversed using SB203580. Nocodazole-induced CK2 activation correlated with increased levels of cyclin B expression and cell cycle arrest, whereas the compromised checkpoint was associated with a reduction in levels of cyclin B and consequently a reduction in cdc2 activation. The latter event was shown to be dependent upon p53. The reduction in apoptosis exhibited by CK2 depleted cells, accompanied by the significantly reduced mitotic index provides the strongest support for the notion that the mitotic checkpoint arrest was breached. The identical cell cycle distribution pattern observed for both nocodazole-treated and AS CK2-depleted $p53^{-/-}$ cells (Figure 23) provides the most compelling evidence against an alternative cell cycle modulating role for depletion of CK2 in mediating the above responses.

The C-terminal domain of p53 is a target for CK2 phosphorylation. CK2 and p53 associate *in vivo* via binding of the regulatory β subunit of CK2 to the C-terminal domain (Appel et al., 1995; Gotz et al., 1999). The effect of this phosphorylation upon the growth suppressing and transactivating activities of p53 is controversial (Hupp et al., 1992; Rolley and Milner, 1994; Mundt et al., 1997). We have shown that protein kinase CK2 is required for p53 to maintain genomic stability in response to spindle damage. We

have found that p53's function during M phase in response to spindle damage, which has previously been demonstrated to prohibit re-duplication of DNA (Notterman et al., 1998) and centrosomes (Fukasawa et al., 1996) without completion of mitosis, was dependent on the direct effect of CK2 on p53. This effect correlated with phosphorylation of the p53 protein at Ser- 392 (Fig. 15).

CK2 was found to function as a component of the mitotic spindle checkpoint as demonstrated in Figures 10, 17 and 18. This was demonstrated by showing that the sustained activation of $p34^{cdc2}$/cyclin B, due to activation of the spindle assembly checkpoint by nocodazole was completely suppressed by either depletion of CK2 catalytic subunits or inhibition of its activity using antisense or DRB, respectively. We have also shown that depletion or inhibition of CK2 did not affect the inhibitory phosphorylation of cdc2 at Thr-14 or Tyr-15, nor the activity of cdc2 by phosphorylating Thr-161 (Fig. 17-18), but did affect the expression of the regulatory cyclin B (Fig. 18).

The question remained as to whether the override of the mitotic arrest, observed in the absence of CK2, might be due to p53-mediated abrogated expression of cyclin B. It has been shown that HeLa cells with a mitotic inhibitor such as nocodazole for 12 to 48 h results in elevated cyclin B levels (Kung et al., 1990; Pines, 1999). These data led to the conclusion that prolonged inhibition of the segregation of chromosomes in human cells is associated with sustained cyclin B levels and mitotic arrest. Hixon et al. (1998) have reported that cells expressing p53 mutants show an unscheduled degradation of cyclin B protein in response to mitotic spindle-depolymerizing drugs. It has been also shown that activation or overexpression of p53 can directly regulate cyclin B expression by lowering the intracellular levels of cyclin B mRNA (Innocente et al., 1999; Taylor et

al., 1999). These data indicated that inactivation of cdc2 kinase through cyclin B repression is an essential step in p53-mediated mitotic arrest. Therefore, we determined the expression of cyclin B protein. Our results showed that the cyclin B expression level (Fig. 21A) was not affected in the HCT116 $p53^{-/-}$ cell line, in the absence or inhibition of CK2, during persistent activation of the mitotic spindle checkpoint by microtubule-destabilizing drugs (in contrast to $p53^{+/+}$ cells Fig. 21B). Furthermore, our cell cycle analysis data showed that cells lacking functional p53 underwent mitotic delay for up to 24 hours (Fig. 19). Although it has been shown that p53 does not affect the initiation, duration or magnitude of the mitotic delay following disruption of the mitotic spindle, it is an essential element of the mitotic checkpoint which prohibits re-duplication of DNA (Notterman et al., 1998).

Previous results have shown that mutation of p53 protein at the CK2 phosphorylation site (Ser-392) inhibited the ability of p53 to cause growth repression but had no effect on its transactivation activity (Hall et al., 1996b). p53 transrepression has been suggested to be required for induction of p53-dependent apoptosis (Sionov and Haupt, 1999).

Via regulation through the carboxyl terminus of p53, it has been suggested that p53 can exist in two conformations with different properties, one configuration might be inhibited for DNA binding but might remain active for other activities (Ko and Prives, 1996).

We thus propose that the depletion of CK2, which causes suppression of p53 protein phosphorylation at Ser-392, may confer upon p53 a gain of oncogenic properties phenotype that results in increased cell survival and post-transcriptional degradation of

cyclin B, compromising the mitotic checkpoint arrest that leads to genomic instability. In other words, p53 may lose the ability to maintain mitotic integrity, in the absence or dysfunction of CK2, by a loss of its ability to appropriately regulate cyclin B levels, as well as an inability to excute apoptosis. This was clearly demonstrated not only in HeLa cells but also in the colorectal MIN cancer cell line, which has an intact spindle checkpoint.

Chromosome instability reflects the occurrence of defective mitosis, including unequal distribution of chromosomes to daughter cells and failure to undergo cytokinesis, which leads to generation of aneuploid cells. Both *in vivo* and *in vitro*, chromosome instability has been shown to correlate with loss or mutation of p53 protein (Fukasawa et al., 1996; Fukasawa, 1997). It has been shown that loss of p53 protein in mouse embryonic fibroblasts results in the acquisition of multiple copies of centrosomes upon nocodazole treatment (Fukasawa et al., 1996; Tarapore and Fukasawa, 2000). These cells have shown to have abnormally amplified multiple copies of centrosomes that co-migrate to the poles, establishing pseudobipolarity (Fukasawa et al., 1996; Tarapore and Fukasawa, 2000). Cells with such pseudobipolar spindles have been shown to undergo normal chromosome segregation and cytokinesis (Tarapore and Fukasawa, 2000). Similarly, our results showed that the loss of CK2 in HeLa cells, exposed to nocodazole, resulted in an abnormal number of centrosomes that failed to position along the bipolar axis (Fig. 24). These cells were also shown to undergo cytokinesis, despite leaving mitosis with a disrupted spindle. Although this result is yet to be verified by genetic manipulation that is ongoing in our laboratory, our results indicate that CK2 may be

involved in a p53-dependent regulation of centrosome duplication in response to spindle damage.

6. CONCLUSION

In this thesis I have shown that protein kinase CK2 may be regulated by the stress-stimulated p38 MAP kinase signaling pathway. This is the first placement of CK2 within a specific signaling pathway. I have showed that upon phosphorylation and activation of p38α by different stress stimuli, it is able to engage CK2 in a complex through direct interaction with CK2 enzyme. Through an allosteric effect exerted by active p38α, CK2 becomes stimulated to phosphorylate other targets such as the tumor suppressor p53 protein. We have shown that stress-activated CK2 mediated by the direct protein-protein interaction with p38 MAP kinase regulates the phosphorylation of the C-terminus of p53 protein at Ser-392. The implication of this phosphorylation is that it may have a potential function in coordination of cellular processes in response to genotoxic stresses such as protein synthesis, mitochondrial function and cell cycle progression. Protein kinase CK2 may have an essential role in maintaining the integrity of cellular function by collaborating with tumor suppressors such as p53. In this report, we have shown that phosphorylation of p53 at Ser-392 was correlated with the regulation of p53 function in response to spindle damage. Our results have demonstrated that the p38-CK2 complex function in concert with p53 to ensure mitotic fidelity. Abrogation of CK2 activity in response to mitotic crisis may confer upon p53 oncogenic properties manifested in cell survival and chromosomal instability. Therefore we hypothesize that under cellular stress, CK2 functions as a tumor suppressor in concert with p53.

Thus I propose a signaling pathway that regulates p53 in the event of mitotic crisis such as spindle damage. Upon microtubule disruption, we have shown that p38

MAP kinase is activated and associates with CK2, regulating CK2's activity and its translocation to the spindle body or centrosomes. This, in turn, exerted a direct effect on the binding and phosphorylation of p53, modifying p53's function to ensure maintenance of genomic fidelity (Fig. 25).

Protein kinase CK2 activity is elevated in a multitude of human cancer cells including leukemias and solid tumors (Pinna and Meggio, 1997; Xu et al., 1999). Seldin and Leder (1995) have demonstrated that CK2 upregulation was critical to the pathogenesis human cancer by overexpressing the catalytic α subunit in transgenic mice. CK2α transgenic mice develop lymphoma beginning at about six months of age with an incidence of 15-20% per year (Seldin and Leder, 1995; Xu et al., 1999). Similarly, overexpression of CK2α in the mammary gland of transgenic mice causes hyperplasia and dysplasia of the female mammary gland (Landsman-Bollag et al., 2001). In addition Landesman-Bollag et al. (1998) have demonstrated that overexpression of CK2α contributed to the loss of p53 alleles. Thus, it has been proposed that CK2 might be a 'proto-oncogene', a gene whose dysregulated or ectopic expression can cause cancer (Seldin and Leder, 1995; Guerra et al., 1999; Xin et al, 1999).

In contrast to the above reports, we hypothesize that protein kinase CK2 may function as a tumor suppressor in cooperation with p53 protein. However, in the event of misexpression of CK2α or CK2β subunit, which is common in most tumors, CK2 may have a negative effect on p53 function that alter its transrepression activity and gain of oncogenic properties. This was mainfested by genomic instability (defective checkpoints and abnormal centrosome amplification) that perhaps contributes to the initiation and progression of tumorigenicity. Finally, this study underscores the potential functional

importance of the association of these two highly altered molecules (CK2 and p53) in human cancer, which clearly merits further investigation.

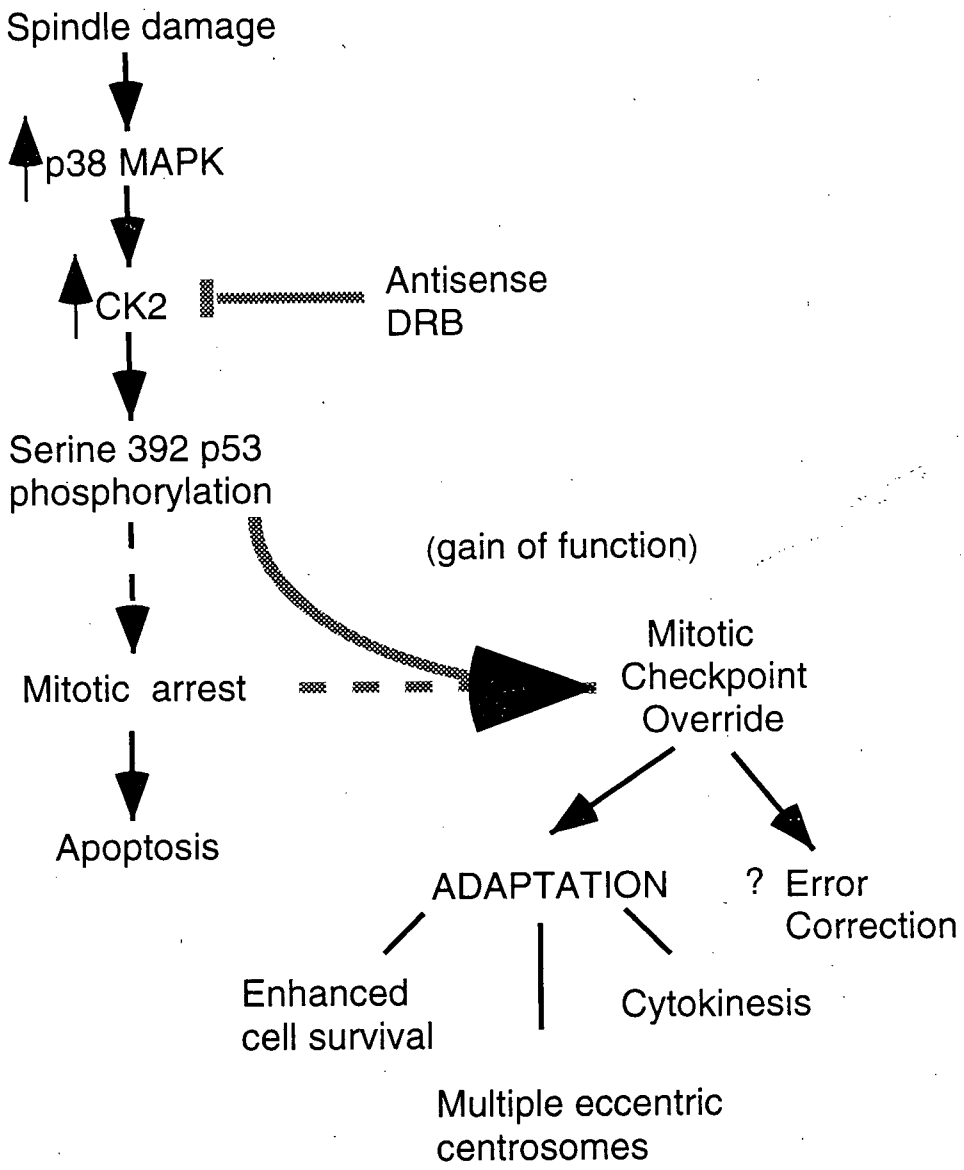

Figure 25: Schematic of events following nocodazole-induced spindle damage. Following activation of p38 MAPK there is a translocation and activation of CK2 to the centrosomes and damaged spindle. This is associated with serine 392 p53 phosphorylation which can be attenuated by pharmacological (DRB) means and antisense depletion of CK2a. Although mitotic arrest is independent of p53, subsequent override is dependent upon p53-mediated cyclin B degradation that requires CK2. Black solid arrows (direct), broken arrows (indirect), indicate events following spindle damage, grey arrows indicate those events following depletion/inhibition of CK2.

7. FUTURE STUDIES

I propose that the holoenzyme CK2 functions as a tumor suppressor. However, it may also fulfill the definition of an oncogene if is mutated, dysregulated or mis-expressed.

Based on my findings I recommend experiments to identify the critical residues mediating the protein-protein interaction between p38 MAP kinase and protein kinase CK2.

This work can be carried out by mapping the interacting binding residues between CK2 and p38 MAP kinase. We have shown that the p38 MAP kinase inhibitor SB203580 interferes with the CK2-p38 complex formation, and it was previously known to disrupt the protein-protein interaction between p38 MAP kinase and its substrates at the ATP binding pocket of p38. Thus, by using site specific mutagenesis, it should be possible to construct a series of deletion mutants as well as defined mutations in the ATP binding pocket of p38 MAP kinase.

I also recommend experiments to investigate the involvement of CK2 in p53-dependent apoptotic response to genotoxic stress (microtubule inhibitors, UV and γ-radiation). This could include assessment of p53-induced genes mediating apoptosis such as Bax. Miyashita and Reed (1995) have shown that p53 is a direct transcriptional activator of the human Bax gene. This work would depend upon the creation of corresponding mutations in protein kinase CK2. The functional significance of this will depend upon assays such as p53 phosphorylation and transcription of p53 dependent genes (this can be done using luciferase-based promoter activation of genes such as Bax).

Several studies from different laboratories including ours indicate that the CK2 catalytic subunit α is disproportionately overexpressed compared with the regulatory β subunit in human cancer. I strongly believe that the aberrant expression of the stable holoenzyme CK2 (or the catalytic component) may play an integral role in human cancer particularly in colon cancer. Therefore, I suggest further investigation whether CK2 subunits are mutated, truncated or genetically mis-expressed in colon cancer.

Experiments could be performed by generating transgenic mice that carry the wild-type CK2 subunits, using an intestine-specific promoter. This could determine whether it possesses oncogenic function within this system.

For the first part, I recommend sequencing the entire coding region of each of the 2 CK2 genes using overlapping PCR both in available colon cancer cell lines and in cancer cells derived from human tumor samples. For the second part, transgenic mice should be generated that express a CK2 transgene using either a villin or Fabp intestinal specific promoter.

The significance of this work is that it will provide an improved understanding of the role of protein kinase CK2 in cancer and possibly provide an important target for gene therapy.

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