EXPRESSION OF EPITOPE TAGGED PROTEIN KINASE CK2 IN MAMMALIAN CELLS

BY

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ZILONG WANG

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

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This manuscript is dedicated to

my parents, Shunfu Ning and Caigao Wang,

my wife, Keqin Tu,

and my son, Gordon Xuefeng Wang

Abstract

Protein kinase CK2 (also known as Casein Kinase II) is a protein serine/threonine kinase. It is an ubiquitously distributed tetrameric enzyme composed of two catalytic subunits (α and/or α') and two regulatory subunits (β). The α and α' are quite similar except their carboxyl termini. The precise role of CK2 in biological responses remains to be characterized, but a great deal of recent evidence indicates that CK2 is involved in the regulation of proliferative events.

There have been indications that CK2 may be regulated by phosphorylation. It has been demonstrated that the α and β subunits of CK2 are dramatically phosphorylated in mitotic cells. CK2β subunit has an autophosphorylation site (Ser2, Ser3) and a mitotic phosphorylation site (Ser209). The mitotic phosphorylation sites on the CK2α subunit (Thr344, Thr360, Ser362 and Ser370) are localized to its unique carboxyl terminus. The mitotic phosphorylation of CK2 occurs to high stoichiometry, indicating that certain functions or properties of CK2 could be regulated by its cell cycle-dependent phosphorylation.

As a first step towards determining whether the phosphorylation of CK2 is important for cell division, Cos-7 cells have been transfected with cDNAs encoding wild type or non-phosphorylatable mutants of CK2α, CK2α' or CK2β. To

discriminate between the transfected CK2α, CK2 α' or CK2β and their endogenous counterparts, the cDNAs have been modified by the addition of different epitope tags at the amino or carboxyl terminus. Transient transfection studies indicate that HA-tagged CK2α, CK2α' wild type and non-phosphorylatable forms behave in a similar manner to non-tagged CK2α and CK2α'. Similarly, myc-tagged CK2β wild type and mutants can bind CK2α subunits to form holoenzyme, in a manner comparable to normal CK2β. Furthermore, immunofluorescent localization studies demonstrate expressed CK2α and CK2α' wild type locate primarily to the nucleus like their endogenous counterparts. All these analyses indicate that the expression constructs are functional and the epitope tags have no effect on the properties of CK2 subunits. The studies described in this thesis are an important prelude to a systematic examination of the functional role of CK2 and its phosphorylation during cell cycle progression.

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ABBREVIATIONS

A	deoxyadenosine
α-MEM	alpha-minimal essential medium
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C	deoxycytidine
^0C	degree centigrade
cDNA	complementary DNA
Ci	curie
CIP	calf intestinal alkaline phosphatase
CK2	Protein Kinase CK2 or Casein Kinase II
CO_2	carbon dioxide
Cos-7	SV40 transformed African green monkey kidney cell line
cpm	counts per second
CsCl	cesium chloride
CTD	carboxyl terminal domain
dATP	2'-deoxyadenosine 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	deoxyguanosine
g	gram; gravity
h	hour
HA	haemagglutinin epitope of influenza virus
HAc	acetic acid
HCl	hydrochloric acid
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid)
H_2O	water
HRP	horse radish peroxidase
IgG	immunoglobulin G
kb	kilobase pair
kDa/Kd	kilodalton
L	litre

LB	Luria-Bertani medium
M	molar
μCi	microcurie
μg	microgram
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
μl	microlitre
μM	micromolar
mM	millimolar
9E10	monoclonal antibody against human c-myc
NaCl	sodium chloride
NaOH	sodium hydroxide
NP-40	Nonidet P-40
O.D.	optical density
%	percent
$p34^{cdc2}$	cyclin dependent kinase 1
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride membrane
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	second
SV40	simian virus 40
12CA5	monoclonal antibody against HA epitope
T	deoxythymidine
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tris-HCl	Tris(hybroxymethyl)aminomethane hydrochloride
TRITC	tetramethylrhodamine B isothiocyanate
U	uridine; unit
v/v	volume per volume
w/v	weight per volume

Amino acids

A/Ala	alanine
C	cysteine

D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S/Ser	serine
T/Thr	threonine
V	valine
Y	tyrosine

Plasmid constructs

CK2α-Wt Plasmid encoding wild type protein kinase CK2α subunit

CK2α-Mt Plasmid encoding mutant form (T/A344, T/A360, S/A362, S/A370) of protein kinase CK2α subunit

α-HA-Wt Plasmid encoding wild type of protein kinase CK2α subunit with C-terminal HA epitope tag

α-HA-Mt Plasmid encoding mutant (T/A344, T/A360, S/A362, S/A370) of protein kinase CK2α subunit with C-terminal HA epitope tag

myc-β-Wt Plasmid encoding wild type protein kinase CK2β subunit, with myc tag

myc-βSer/Ala209 Plasmid encoding myc tagged protein kinase CK2β subunit with a mutation of its mitotic phosphorylation site Serine209 to Alanine209

myc-βΔ1-11 Plasmid encoding myc tagged protein kinase CK2β subunit with a deletion of residues 1-11 including the autophosphorylation sites at Serine 2 and Serine 3

myc-βΔ1-11Ser/Ala209 Plasmid encoding myc tagged protein kinase CK2β subunit with a deletion of its autophosphorylation sites (Serine 2 and Serine 3) and a mutation of its mitotic phosphorylation site Serine 209 to Alanine 209

1. INTRODUCTION

Protein phosphorylation and dephosphorylation play a cardinal role in regulating many cellular processes relating to the control of cell growth and proliferation. Genetic and biochemical studies have demonstrated the existence of protein kinase cascades that are involved in the regulation of cell division and in the transmission of regulatory signals that control cell cycle entry and progression (Reviewed by Hunter, 1995; Pelech et al, 1993; Norbury and Nurse, 1992; Hunter and Karin, 1992; Forsburg and Nurse, 1991; Pelech et al, 1990). The protein kinase cascades allow the amplification, feedback, cross-talk, and branching of signals in the cell signalling process. One component of protein threonine/serine kinase cascades is protein kinase CK2 (also known as Casein Kinase II) that is ubiquitously distributed in the nucleus and cytoplasm of all eukaryotic cells (reviewed by Pinna, 1997; Allende and Allende, 1995; Litchfield and Luscher, 1993; Tuazon and Traugh, 1991; Meisner and Czech, 1991; Pinna, 1990; Krebs et al, 1988). While its precise role remains to be defined, a number of recent studies have demonstrated that protein kinase CK2 plays an important role in the regulation of proliferative events of cells.

1.1 Structure of CK2

The holoenzyme that has been purified from many different eukaryotic cells is generally a tetramer with a molecular weight approximately of 130 kDa. This

tetramer is composed of two types of structurally analogous α (42-44 kDa) or α' (38 kDa) subunits and two β (26 kDa) subunits which associate to form $\alpha_2\beta_2$, $\alpha'_2\beta_2$ or $\alpha\alpha'\beta_2$ structures (Chester et al, 1995; Gietz et al, 1995). The α and α' have been identified as the catalytic active subunits responsible for nucleotide binding and phosphoryl transfer (Bodenbach et al, 1994; Hathaway et al, 1981). The β subunit is a regulatory subunit which up-regulates the activity of the catalytic subunit and affects its substrate specificity (Allende and Allende, 1995; Bidwai et al, 1993).

Molecular cloning studies have demonstrated that the α and α' subunits are coded by different genes (Maridor et al, 1991; Lozeman et al, 1990; Padmanabha et al, 1990; Chen-Wu et al, 1988). Deduced amino acid sequence analysis indicates that both catalytic subunits have all the conserved subdomains of protein kinase family members (Hanks et al, 1991). In mammals and birds, the α (391 amino acids) and the α' (350 amino acids) subunits display a very high degree of identity. Human α and α', for example, have a homology of nearly 85% between their deduced amino acid sequences (Lozeman et al, 1990). Interestingly, the carboxyl terminal domains of the α and α' subunits are completely different sequences, indicating that distinct functions between these two isoforms may be due to divergences within these domains (Litchfield and Luscher, 1993). Amino acid sequences of these two isoforms indicate high evolutionary conservation among species. The homology between chicken and human α subunits is 98% identity while the α' subunits from

species exhibit 97% identity (Litchfield and Luscher, 1993; Maridor et al, 1991; Lozeman at al, 1990). On the other hand, the β subunit of CK2 does not exhibit extensive homology with any other proteins of known function, but is remarkably conserved in different species. Astonishingly, the deduced sequences of human (Jakobi et al, 1989; Teitz et al, 1990), mouse (Boldyreff et al, 1991; Kopatz et al, 1990) and chicken (Maridor et al, 1991) β are identical.

The interactions between CK2 subunits have been studied in several laboratories by using yeast two-hybrid system (Fields and Song, 1989). Gietz et al (1995) reported that the α and α' subunits can interact with the β subunit, but not with other α or α' subunits. In contrast, the β subunits can interact with the α, α' or β subunits, therefore the dimerization of the β subunits brings two heterodimers (αβ or α'β) into a tetrameric complex of CK2. Kusk et al (1995) demonstrated that the divergent C-terminal domain (from amino acid residues 351 to 391) of the α subunits is not required for interaction with the β subunits and that the sequence of the amino acid residues 152 to 200 in the β subunits contains the minimal domain critical for interaction with the α subunits. Delineation of the domain responsible for β subunit dimerization has not been definitively completed. The studies from Kusk et al (1995) suggested that the dimerization domain is located within amino acid residues 20 to 145 in the β subunit. In contrast, Boldyreff et al (1996) reported that the important region for successful dimerization of the β subunit is located to amino acid residues

156-165. Furthermore, the study also demonstrated that the amino acid residues between 170 and 180 antagonize the dimerization of β subunits. Further study is required to resolve the precise residues that control β subunit dimerization. Also, residues on α or α' that are involved in interactions with CK2β remain to be defined.

1.2 CK2 in signal transduction

CK2 is a serine/threonine kinase found in all eukaryotic cells. Gene disruption studies in yeast and in *Dictyostelium discoideum* demonstrated that CK2 is essential for cell viability (Kikkawa et al, 1992). It has been demonstrated that disruption of *Saccharomyces cerevisiae* CK2α gene (CKA1) or CK2α' gene (CKA2) alone does not yield a detectable phenotype, but simultaneous disruption of both α and α' genes is lethal (Chen-Wu et al, 1988; Padmanabha et al, 1990; Bidwai et al, 1992). In the latter case, the phenotype can be restored by introduction of cDNA encoding a functional *Drosophila* CK2α subunit, but not a kinase-inactive CK2α subunit (Birnbaum et al, 1991). Disruption of fission yeast *Schizosaccharomyces pombe* CK2α gene also demonstrated that this catalytic subunit is required for cell morphogenesis and maintenance of polarized growth (Snell and Nurse, 1994). Kikkawa et al (1992) reported a failure to achieve disruption of the CK2α gene in the

cellular slime mode *Dictyostelium discoideum*, suggesting that this gene is essential for vegetative growth of this organism. Taken together, all the data suggest the catalytic activity of CK2 is essential for cell growth.

The important role of CK2 in signal transduction also comes from the evidence that CK2 phosphorylates regulatory proteins in the cytoplasm and the nucleus where this enzyme is distributed. Phosphorylation of the regulatory nuclear proteins especially transcription factors by CK2 has been suggestive of a role for CK2 in the control of cell proliferation (Allende and Allende, 1995; Litchfield and Luscher, 1993). In some instances, phosphorylation of transcription factors by CK2 has been shown to inhibit the DNA binding activity, including c-Myb (Luscher et al, 1990), c-Jun (Lin et al, 1992), Max homodimer (Berberich and Cole, 1992), Sp1(Armstrong et al, 1997). In other situations, phosphorylation by CK2 leads to activation of gene transcription factors such as the NF-Kappa/Rel-IKappaB complex (Schwarz et al, 1996; Berroga et al, 1995), and p53 (Prowald et al, 1997; Hupp et al, 1992). Collectively, these results indicate that CK2 is an important component in the signalling pathways leading to proliferation.

1.3 CK2 in cell cycle progression

A number of observations have demonstrated that CK2 plays an important role

at different stages of the cell cycle. The involvement of CK2 at the early stage of the cell cycle comes from the evidence that mitogenic stimulation of quiescent cells resulted in a striking shift of the intracellular distribution of CK2 toward an increased nuclear concentration (Krek et al, 1992; Gauthier-Rouviere et al, 1991; Filhol et al, 1990; Belenguer et al, 1989). Gauthier-Rouviere et al (1991) reported that the activation of quiescent rat embryo fibroblast cells by serum was accompanied by the nuclear translocation of endogenous CK2 from the cytoplasm. A related observation that microinjection of anti-CK2β antibodies into cells caused significant inhibition of growth stimulation was also reported (Lorenz et al, 1994). Furthermore, incubation of quiescent cells with oligonucleotides directed against CK2α or CK2β resulted in the failure of cell progression into S phases upon mitogen stimulation (Lorenz et al, 1994; Pyerin et al, 1992). Collectively, those results indicate that an increase of CK2 and translocation to the nucleus are required for cell cycle progression.

Altered CK2 activity in tumour cells also indicates it may play a role during cell cycle progression. The activity of CK2 was dramatically increased in some human leukaemia (Friedrich and Ingram, 1989), in solid tumours (Munstermann, 1990), and in head and neck carcinoma compared to the normal mucosa cells (Gapany et al, 1995) suggesting that dysregulation of CK2 may have an important role in the tumorigenesis. Elevated CK2 activity was also observed in the transformed cells that grow faster than non-transformed cells (Daya-Makin et al, 1994).

Transgenic studies have demonstrated that the tumorigenic processes induced by transcription factors such as c-myc (Seldin and Leder, 1995) and Tal-1 (Kelliher et al, 1996) have been dramatically enhanced with co-expression of CK2α subunit in transgenic mice. It is noteworthy that these transcription factors are protein substrates of protein kinase CK2. These results suggest that increased activity of CK2 in tumour cells may contribute to tumorigenesis.

Further support for a role of CK2 in cell cycle progression comes from a study using Daidzein which inhibits the activity of CK2 but not MAP2 kinase. Daidzein treatment of Swiss 3T3 cells resulted in inhibition of S phase progression induced by insulin or insulin-like growth factor stimulation (Higashi and Ogawara, 1994). Gene disruption studies and studies with temperature sensitive alleles of CK2α in *Saccharomyces cerevisiae* demonstrate that CK2 is necessary for viability and that CK2 is required for G1/S and G2/M transition of cell cycle (Hanna et al, 1995; Reed et al, 1994; Cardenas et al, 1992).

Overall, all the above evidence clearly indicates CK2 plays an important role in controlling cell proliferation. The precise role(s) of CK2 and how its functions are controlled during the cell cycle remain to be defined.

1.4 Phosphorylation of CK2 and cell cycle progression

Phosphorylation may play an important role in the regulation of CK2 in cells. Ackerman et al (1990) reported that CK2 is activated in epidermal growth factor (EGF) treated human A-431 carcinoma cells and suggested that stimulation of the kinase resulted from an elevation of its phosphorylation. *In vitro*, treatment of CK2 with purified protein kinase C (Sanghera et al, 1992) or purified $p34^{cdc2}$ (Mulner-Lorillon et al, 1990) also leads to the phosphorylation and activation of CK2. In contrast, Agostinis et al (1987) demonstrated that dephosphorylation of purified CK2 by protein phosphatases resulted in an increase of CK2 activity. Overall, these reports indicate that CK2 may be regulated by phosphorylation. However, there are clearly discrepancies regarding the role of phosphorylation in regulating CK2 activity since the sites that are phosphorylated in cells in response to EGF, or the sites that are affected by protein kinase C, $p34^{cdc2}$ or protein phosphatases were not determined in any of these studies. To systematically examine the role of phosphorylation in regulating the cellular function of CK2, Litchfield et al (1991) identified the sites on the β subunit of CK2 that are phosphorylated in cells. By using metabolically labelled human epidermal carcinoma A431 cells Litchfield et al (1991) demonstrated that β subunit of CK2 is phosphorylated at an autophosphorylation site (Serine2, Serine3) and at a residue (Serine209) that is phosphorylated *in vitro* by $p34^{cdc2}$. Subsequently,

results from Boldyreff et al (1993) confirmed the autophosphorylation of CK2β at Serine2 both on bacterially expressed recombinant human CK2β subunit and the CK2β isolated from native rat liver. Further study by Litchfield et al (1992) indicated that the α (but not the α') subunit and the β subunit of CK2 are dramatically phosphorylated in cultured chicken BK3A cells and human Jurkat cells arrested at mitosis by nocodazole treatment. Comparative phosphopeptide mapping indicated that CK2 is a likely physiological substrate of $p34^{cdc2}$ in cells. Since the activity of CK2 was not observed to be increased by mitotic phosphorylation, the phosphorylation of CK2 may not have a direct impact on the catalytic properties of CK2 (Litchfield et al, 1992). Nevertheless, there are numerous indications that suggest that the mitotic phosphorylation of CK2 could be important. Phosphorylation of CK2 is high stoichiometry and involves multiple sites. Also the phosphorylation sites are highly conserved between chicken and humans since the phosphopeptide maps are nearly identical. Importantly, only one isoform of CK2 is phosphorylated; CK2α but not CK2α' is phosphorylated. There are additional observations that suggest CK2 has a role in mitosis. Immunocytochemical studies by Yu et al (1991) initially indicated that CK2 is associated with spindle fibers during metaphase and anaphase of mitosis. Similarly, Krek et al(1992) examined the subcellular localization of CK2 in three different types of cells and observed that CK2 is associated with the mitotic spindle independent of the cell type analysed.

Based on these results, we hypothesized that CK2 is a regulatory participant in the protein kinase cascades that control cell cycle progression and that the phosphorylation of CK2 is required for cell division. Towards the objective of testing this hypothesis, the cDNA encoding CK2α was subcloned into a vector with a constitutive CMV promoter (ie pRc/CMV from Invitrogen). In order to distinguish the transfected CK2α from the endogenous CK2α, an epitope tag (the HA tag of influenza virus (Egan et al, 1993; Tyers et al, 1992; Wilson et al, 1984)) that can be detected by 12CA5 antibodies (Berkeley Antibody Company) was fused to either the N-terminus or the C-terminus of CK2α. Since the mitotic phosphorylation sites of CK2α have been identified by Bosc et al (1995), a non-phosphorylatable form of CK2α was also created. In this mutant, each of the $p34^{cdc2}$ phosphorylation sites on CK2α was mutated to a non-phosphorylatable alanine residue (Thr344/Ala, Thr360/Ala, Ser362/Ala, Ser370/Ala). As with the wild-type CK2α construct, an epitope tag was incorporated at the N-terminus of the coding sequence to facilitate detection of the expressed protein. Similar plasmid constructs were created for the CK2β subunit. A human myc epitope tag was fused to the N-terminus of CK2β to permit detection of exogenous CK2β by 9E10 monoclonal antibodies produced by a hybridoma cell line obtained from ATCC even in the presence of endogenous CK2β in the cell. Mutant forms of CK2β were created by the deletion of a small region of residues including its autophosphorylation sites Ser2 and Ser3 and also by mutation

of its mitotic phosphorylation site Ser209 into Ala209.

In order to study the expression of CK2 in cells, the Cos-7 cell line (ATCC) was chosen as the expression system. Cos-7 (Gluzman, 1981) is a SV40 virus transformed green monkey kidney cell line with a relatively short doubling time, that is easy to be cultured. Cos-7 cells are a suitable host for transient transfection of an eukaryotic expression vector with a SV40 origin, such as pRc/CMV which was used in my study, since this cell line constitutively produces SV40 large T antigen which stimulates the episomal replication of the plasmid.

In this thesis, I presented data describing the construction of plasmids encoding wild type and mutants of CK2α and CK2β subunits and the establishment of optimal conditions of transient transfection in Cos-7 cells. Ultimately the expressed CK2 was used to test the kinase activity, holoenzyme formation between the subunits of CK2 and subcellular localization of some of the expressed proteins. This study will ultimately benefit further study of the role of mitotic phosphorylation of CK2 on cell cycle progression.

2. MATERIALS AND METHODS

2.1 Materials

Restriction enzymes and DNA and RNA modifying enzymes were obtained from Pharmacia (Canada) Inc. (Baie d'Urfe, Quebec, Canada), New England Biolabs Inc. (Mississauga, Ontario, Canada), or Gibco/BRL (Burlington, Ontario, Canada). The TA^R cloning kit and the expression vector pRc/CMV kit were purchased from Invitrogen Corporation (San Diego, California, USA). ^{32}P-γATP and ^{35}S-dATP were from Amersham Canada Ltd. (Oakville, Ontario, Canada) or from ICN Biochemicals Canada Ltd. (Mississauga, Ontario, Canada). Oligonucleotides were synthesized by Dr. M. Mowat (Manitoba Institute of Cell Biology, Winnipeg, Manitoba, Canada) or were obtained from Dalton Chemical (Toronto, Ontario, Canada) or from Gibco/BRL (Burlington, Ontario, Canada). The Bst^R DNA sequencing kit was purchased from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario, Canada) and the T_7 DNA sequencing kit was obtained from Pharmacia (Canada) Inc. The Prep-A-Gene DNA purification kit, nitrocellulose and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad. SV40 transformed African green monkey cells (Cos-7) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). All tissue culture media and solutions were obtained from Gibco/BRL. Fetal bovine serum was obtained from Intergen (Purchase, NY, USA). Other chemicals and reagents were of reagent grade.

2.2 Cell lines and cell culture

2.2.1. Cell lines

The Cos-7 cell line is an African green monkey fibroblast-like kidney cell line established by Gluzman (1981) from CV-1 simian cells which were transformed by an origin-defective mutant of SV40 encoding wild-type T antigen. Cos-7 cells constantly express SV40 T antigen which stimulates the replication of plasmid vectors with a SV40 origin, such as the expression vector pRc/CMV used in this study.

2.2.2 Cell Culture

Cell lines were grown in 100x20mm disposable sterile tissue dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA; Sarstedt Inc., St-Leonard, Quebec, Canada) and maintained in a constant environment of 37 °C, 5% CO_2/95% air and 100% humidity. Tissue culture media were replaced every 48-72 hours. Cos-7 cells were maintained in α-minimal essential medium (α-MEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were visualized with phase contrast optics using a Zeiss inverted microscope

(Carl Zeiss, D-7082 Oberkochen, Germany).

Cultures, near complete confluence, were harvested from tissue culture dishes by rinsing with 10 ml of calcium and magnesium free phosphate buffered saline (PBS), followed by room temperature incubation in 1 ml of a 0.25% trypsin, 1 mM EDTA solution for 2-3 min. Media (10 ml) was added to each dish and cells removed from the dish by repeated manual pipetting with a sterile pipette. This solution was transferred to a sterile 15 ml polystyrene tube and cells pelleted by centrifugation (1000 rpm or 200 x g). The trypsin/media solution was vacuum aspirated into a waste bottle. Cells were resuspended in culture media, cell numbers were determined using a haemocytometer and cells replated on fresh plates. Alternatively, to freeze cells for storage, cells were resuspended in freezing media (50% (v/v) FBS, 10% (v/v) dimethyl sulfoxide (DMSO), 40% (v/v)culture media) at a final concentration of $1\text{-}2 \times 10^6$/ml. Resuspended cells in a volume of 1.8 ml were transferred to Nunc cryogenic vials and stored at -80°C.

Recovery of cell lines from frozen stocks was done by thawing the vial briefly in a 37 °C water bath. Cells were transferred to a 15 ml polystyrene tube and pelleted as described above. The freezing solution was vacuum aspirated and cells were resuspended in the appropriate growth media.

2.3 Antibodies

Polyclonal anti-CK2α antibody was prepared as described (Litchfield et al, 1991) and was directed against the synthetic peptide $\alpha^{376-391}$ (ANPLGMPVPAAAGAQQ). Polyclonal anti-CK2α' antibody directed against the synthetic peptide $\alpha'^{333-350}$ (SQPCADNAVLSSGLTAAR) was also described previously (Litchfield et al, 1992). Polyclonal anti-CK2β antibody against the synthetic peptide $\beta^{198-215}$ (QLQLQAASNFKSPVKTIR) was prepared as described (Litchfield et al, 1991). The monoclonal antibody 12CA5 against the hemagglutinin influenza (HA) epitope was purchased from BAbCO (Berkeley Antibody Company) (Berkeley, CA, USA). The hybridoma producing the 9E10 monoclonal antibodies directed against the myc epitope (Evan et al, 1985; Munro et al, 1987) was from ATCC. Hybridoma cells were injected into mice, ascites fluid was collected and purified by ammonium sulphate precipitation (Harlow and Lane, 1988). Horse radish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad. FITC conjugated goat anti-mouse antibody and TRITC (tetramethylrhodamine B isothiocyanate) conjugated goat anti-rabbit antibody were from Sigma.

2.4 Basic Molecular Biology Methods

2.4.1 Small scale preparations of plasmid DNA

Small scale preparations of plasmid DNA was according to the alkali lysis method as described by Sambrook *et al* (Sambrook et al, 1989).

2.4.2 Large scale preparation of plasmid DNA

The method used was based on the procedure of Sambrook *et al* (Sambrook et al, 1989) with the following modifications. After isolating plasmid DNA from the bacterial culture by the alkali lysis methods, the DNA was purified by double banding on CsCl gradients. Briefly, for each ml of the DNA solution in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 1 g of solid CsCl was added and dissolved. Then 0.8 ml of 10 mg/ml ethidium bromide was added to every 10 ml of DNA/CsCl solution. The sample was centrifuged at 8000 rpm for 5 min and the supernatant was carefully transferred into a Beckman quick-seal centrifuge tube. The tube was placed in a Beckman TLN100 rotor and centrifuged at 100, 000 rpm (20 °C) for 4 h.

Typically, the plasmid band could be visualized under normal light conditions. Plasmid bands that were difficult to detect were visualized by fluorescence using short wavelength ultraviolet light. Plasmid was recovered by inserting a 16 gauge

needle, fitted to a 1 ml syringe, through the centrifuge tube below the plasmid band and gently drawing out the plasmid DNA. The DNA solution was transferred into another fresh Beckman quick-seal centrifuge tube and the tube was topped with a CsCl solution (a ratio of 1 gram CsCl to 1 ml TE). The tube was recentrifuged in a Beckman TLN100 rotor at 100, 000 rpm (20 °C) for 4 hours. The plasmid DNA was collected as described above. Ethidium bromide was removed from the DNA solution by extraction of the DNA solution several times with equal volume of isopentyl alcohol until there was no pink colour seen in the upper organic phase. The DNA was precipitated by the addition of 2 volumes of water and 6 volumes of ethanol followed by incubation on ice for 15 min. The plasmid DNA was then centrifuged for 15 min (4 °C) at 5000 rpm, washed with 70 % ethanol, and resuspended in sterile 0.1 x TE to a final concentration of 2.5 or 5 μg/μl. The integrity of the DNA was assessed using agarose gel electrophoresis and appropriate restriction endonuclease digestion.

2.4.3 Restriction Enzyme Digestion and Agarose Gel Electrophoresis

DNA samples were routinely digested with restriction endonucleases according to manufacturer's specifications. Typically, digestion reactions contained the desired quantity of DNA, a restriction digestion buffer, the restriction endonuclease, and deionized H_2O in a total volume between 20-100 μl depending on

the amount of DNA being digested. Digestion was usually conducted at 37 °C for 1 hour.

Electrophoretic analysis of DNA was done essentially as described by Sambrook *et al* (1989). Depending on the size of the DNA to be visualized or processed, agarose gels between 0.7 % - 2.0 % (w/v) were prepared by dissolving agarose in 40 mM Tris-HAc pH 8.5, 1 mM EDTA (1 x TAE) solution containing 0.4 μg/ml ethidium bromide. The DNA samples containing loading buffer (0.125% (w/v) bromophenol blue, 0.125% (w/v) xylene cyanol, 12.5% (w/v) Ficoll), were loaded into wells and gels were run at a voltage between 60-80 V for 1-2 h. The DNA bands were visualized on a transilluminator.

2.4.4 Isolation of Plasmid DNA Fragments

DNA fragments were isolated from agarose gels using Prep-A-Gene Matrix (Bio-Rad) according to the manufacturer's manual. After plasmids were digested with the appropriate restriction endonuclease and the DNA fragments were separated by agarose gel electrophoresis, the piece of agarose gel containing the DNA fragment of interest was removed and cut into small slices and placed into 1.5 ml microfuge tube. The gel slices were dissolved in 3 volumes of the binding buffer (6 M $NaClO_4$, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) with incubation for 10 min at 37 °C. An

appropriate volume of Prep-A-Gene matrix was added to the gel solution. The sample was then incubated for 10 min at room temperature with end-over-end mixing. The matrix was washed three times with the binding buffer, three times with the wash buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA), and dried in a Speed-Vac concentrator. A small volume of the elution buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was added to the matrix and incubated for 5 min at 37 °C. The eluted solution containing the plasmid fragment was collected by centrifugation of the matrix. The concentration of the fragment was estimated by agarose gel electrophoresis.

2.4.5 Plasmid DNA-Insert Ligation

Plasmid DNA was digested with the restriction enzyme of choice. When it was necessary to dephosphorylate the vectors, the DNA was incubated with calf intestinal alkaline phosphatase (CIP). To the DNA solution, 10 x CIP buffer (0.5 M Tris-HCl pH 8.5, 1 mM EDTA) was added to a final concentration of 1 x CIP buffer followed by the addition of CIP (0.01 units of CIP per pmol of DNA 5'-protruding ends). The reaction mixture was incubated for 30 min at 37 ^{0}C, the sample was then subjected to agarose gel electrophoresis, and the DNA was isolated and purified as described above.

Ligations were performed using vector and insert in a ratio of 1:3 in 1x ligation buffer (6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 0.1 mg/ml bovine

serum albumin, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine), with 2 units of T_4 DNA ligase in a total volume of 10 μl. The ligation reaction was incubated for at least 4 h at 12-14 ^{0}C. *E. coli* competent cells were transformed by either the $CaCl_2$ heat-shock method or by electroporation (Sambrook et al, 1989).

2.4.6 Oligonucleotide-directed mutagenesis

Oligonucleotide directed mutagenesis was used to generate non-phosphorylatable CK2α and CK2β mutants in this study (Kunkel, 1985; Kunkel et al, 1987). The MUTA-GENE^R M13 *in vitro* mutagenesis kit (Bio-RAD) was employed according to the manufacturer's manual.

a. Preparation of uracil-containing single-stranded DNA templates

cDNAs of interest were subcloned into plasmid vectors with fl origin for rescue of single-stranded DNA such as M13 vectors and pRc/CMV vector. *E. coli* CJ 236 cells (*dut$^-$ ung$^-$* F') were transformed with the vectors carrying the cDNAs of interest and the bacteria were cultured in uridine containing LB medium until the O.D. 600 nm reached 0.3. Then M13K07 helper phage was added to the cultures with a ratio of 1/20 of bacteria to phage and the cultures were incubated for 1.5 h at 37°C followed by the addition of kanamycin to a final concentration of 70 μg/ml and

incubation for another 8 h. Phage were harvested by centrifugation of the cultures at 5000 rpm for 10 min and collecting the supernatant. Single stranded uracil-containing DNAs were purified from the phage and verified on agarose gel electrophoresis, and used as templates for further manipulation.

b. Primer extension and transformation of *E. coli* MV1190

Each mutagenic primer was phosphorylated by T_4 polynucleotide kinase and annealed to its corresponding DNA template in an annealing buffer (200 mM Tris, pH 7.4, 20 mM $MgCl_2$, 500 mM NaCl) that was gradually cooled from 70 °C to 30 °C in 40 min. A control was set for each DNA template as the annealing reaction without the mutagenic primer. The synthesis of mutant primer containing DNA strands was completed with the help of T_4 DNA ligase and T_7 DNA polymerase. *E. coli* MV1190 competent cells were transformed with the reaction mixtures including the controls and the bacteria cultures were replated on LB plates.

c. Screening of mutant transformants and DNA sequencing

Individual sterile nitrocellulose membrane discs (0.45 Micron, 82 mm in diameter) were placed on each of the LB plates with transformed *E. coli* MV 1190 colonies to lift bacterial colonies. The membranes were then placed with the bacteria side up on Whatman No. 1 filter papers saturated with 10% SDS for 3 minutes, denatured with 0.5 N NaOH/1.5 M NaCl for 5 min, neutralized with 1.5 M NaCl/0.5 M Tris pH 7.4 for 5 min followed by washing with 2x SSC (0.03 M sodium citrate pH 7.0, 0.3 M NaCl) for 5 min. After air drying for 30 min, the

membranes were dried under vacuum at 80 ^{0}C for 1.5 h followed by soaking them in 2x SSC/0.1% SDS at 37 ^{0}C and washing off the bacterial debris. The membranes were prehybridized with prehybridization buffer (0.9 M NaCl, 0.09 M sodium citrate pH 7.0, 0.1 % (w/v) Ficoll, 0.1% polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 100 μg /ml sheared, denatured salmon sperm DNA) at 55 ^{0}C overnight followed by 65 ^{0}C for 1 h and then hybridized at proper temperature (decided by the melting temperature of the mutagenic primer minus 10 ^{0}C) for 4 h with the prehybridization buffer containing the mutagenic primer labelled with ^{32}P to a final concentration of 10^7 cpm/ml. The membranes were washed with 6x SSC, using incremental increases in temperature of 3 ^{0}C, until there was no detectable signal on the control membranes which carried the bacterial colonies from the reaction without mutagenic primer. At each temperature, the membranes were washed for 2 min and wrapped with Saran Wrap followed by exposure onto a Phosphorimager screen for 50 min. The positive colonies on LB plates were identified by the strong hybridization signals generated by their corresponding colonies on membranes compared to the control membranes. The positive colonies were further verified by DNA sequencing.

2.5 Plasmid Constructs

2.5.1 CK2α and CK2α' constructs

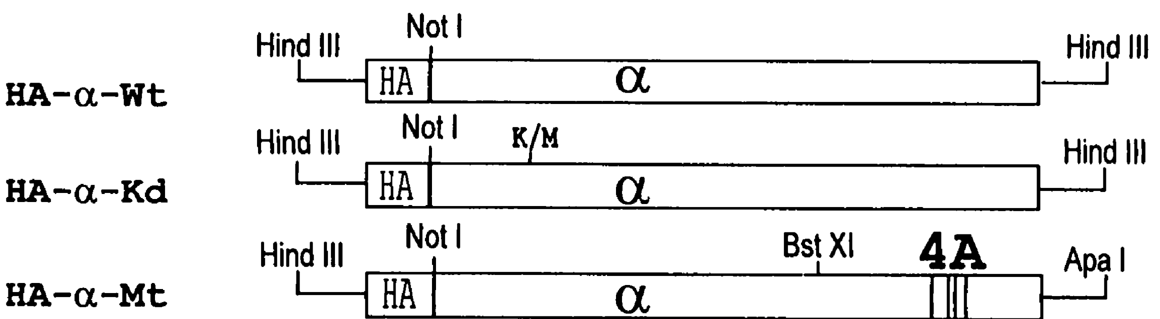
2.5.1.1 HA tagged CK2α wild type (HA-α-Wt) and CK2α' wild type(HA-α'-Wt)

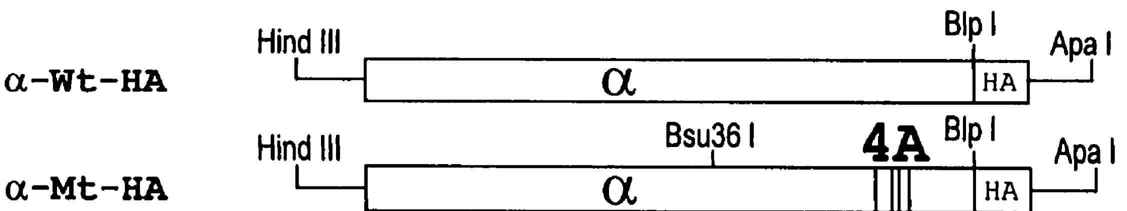
In order to distinguish the expressed protein kinase CK2 from endogenous cellular CK2, the sequences of the catalytic subunits of CK2 (CK2α and CK2α') were modified at their amino termini by the addition of influenza haemagglutinin epitope (HA) tag which can be recognized by the specific antibody 12CA5 (Figure 1, Panels A and C). To achieve this objective, the cDNAs encoding human CK2α and CK2α' (hT4.1 and hT9.1 respectively, Lozeman et al, 1990) were modified by creating a *Not* I site at the 5' end of the two sequences using the polymerase chain reaction (PCR). The initiator methionines of the two sequences were replaced. The primers used for the amplification of CK2α were 5'-ATAAGAATGCGGCCGCTCGGGACCCGTGCCA-3' (sense) and 5'-ATCATAATTGTCATGTCCATGGAA-3' (antisense), and for the amplification of CK2α' the primers were 5'-ATAAGAATGCGGCCGCCCCGGCCCGGCCGCG-3' (sense) and 5'-CAGGATCTGGTAGAGTTGC-3' (antisense). The underlined sequences in the above sense primers indicate the *Not* I sites. The amplified

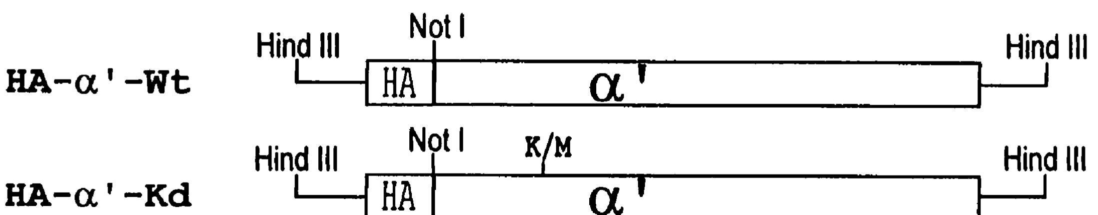
fragment of CK2α encompassed the 5'-end of the modified cDNA to nucleotide 878 while the amplified fragment of CK2α' extended from its modified 5' end to nucleotide 588. The PCR products were digested with *Not* I and *Nco* I for CK2α or with *Not* I and *Ppum* I for CK2α' and were used to replace the respective *Not* I/*Nco* I and *Not* I/*PpuM* I fragments in the hT4.1 and hT9.1 plasmids. The DNA sequence encoding the HA epitope (5'-GGCCGCATCTTTTACCCATACGATGTTCCTGAC TATGCGGGCTATCCCTATGACGTCCCGGACTATGCAGGATCCTATCCA TATGACGTTCCAGATTACGCTGCTAGTGCGGCCGC-3') was kindly provided by Dr. Sean Egan (Hospital for Sick Children, Toronto, Canada) and was modified by the introduction of a start codon and a *Hind* III site at its 5' end using PCR (Innis et al, 1990; Egan et al, 1993). The following primers were used: 5'-CCCAAGCTTCCACCATGGGCCGCATCTTTTACCCA-3' (sense) and 5'-ATAGTTTAGCGGCCGCACTGAGCAGC-3' (antisense). The amplified sequence was directly subcloned into the TA cloning vector (Invitrogen). All amplified sequences were verified by DNA sequencing (Sanger et al, 1977). The TA vector containing the HA epitope-coding sequence was digested with *Not* I and *Apa* I and *Not* I/*Apa* I fragments encoding the modified cDNAs of CK2α and CK2α' were ligated into this vector. The HA-tagged CK2α or CK2α' cDNAs were subsequently subcloned into the *Hind* III site of pRc/CMV (Invitrogen), designated HA-α-Wt and HA-α'-Wt (Figure 1, Panels A and C).

Figure 1. HA tagged CK2α, CK2α' and derivatives

Constructs, designated HA-α (Panel A), α-HA (Panel B) and HA-α'(Panel C) encoding CK2α and CK2α' with HA epitope at the N-terminus (HA-α, HA-α') or C-terminus (α-HA) of each of the proteins were prepared as described in Materials and Methods. As described in the text, a *Not* I site facilitated the ligation of the HA epitope sequence to the cDNAs encoding CK2α and CK2α' (HA-α, HA-α'), as did a *Blp* I site for α-HA constructs. Kinase inactive variants of the two proteins, designated HA-α-K.d. and HA-α'-K.d., were generated by mutation of an essential lysine residue (K68 in CK2α, K69 in CK2α') within the ATP-binding domain of the respective proteins to methionine. Non-phosphorylatable variants of CK2α, designated HA-α-Mt and α-HA-Mt were generated by mutation of mitotic phosphorylation sites at Threonine 344, Threonine 360, Serine 362 and Serine 370 within the C-terminal domain of CK2α into Alanine 344, Alanine 360, Alanine 362 and Alanine 370. Protein encoding sequences derived from CK2α are marked by the open boxes, those derived from CK2α' are marked by the shadowed boxes. The CK2α and CK2α' constructs were subcloned into the *Hind* III or *Hind* III/*Apa* I sites of pRc/CMV. The HA-epitope is recognized by 12CA5 monoclonal antibodies. Polyclonal anti-CK2α antibodies and polyclonal anti-CK2α' antibodies recognize the . carboxyl-terminal domains of the respective proteins.


A
HA-α-Wt
HA-α-Kd
HA-α-Mt
Hind III
Not I
HA
α
K/M
Bst XI
4A
Apa I


B
α-Wt-HA
α-Mt-HA
Hind III
Bsu36 I
4A
Blp I
HA
Apa I
α


C
HA-α'-Wt
HA-α'-Kd
Hind III
Not I
K/M
HA
α'

2.5.1.2 HA tagged CK2α mutant (HA-α-Mt)

The mitotic phosphorylation sites (Bosc et al, 1995) within the carboxyl terminal domain (CTD) of CK2α were mutated into alanine residues (T344/A, T360/A, S362/A, S370/A) by oligonucleotide-directed mutagenesis. A *Sph* I/*Eco* RI fragment encoding this CTD was used to replace the *Sph* I/*Eco* RI portion of the wild type CK2α CTD in hT4.1. A *Bst* XI/*Apa* I fragment was obtained from this construct and used to replace the corresponding portion of the wild type CTD of HA-tagged CK2α in pRc/CMV. The resulting expression vector is designated as HA-α-Mt (Figure 1, Panel A). The replaced region was verified by DNA sequencing (Sanger et al, 1977).

2.5.1.3 CK2α and CK2α' kinase inactive mutants (HA-α-K.d., HA-α'-K.d.)

To create the kinase inactive mutants of HA-α and HA-α', designated as HA-α-K.d. and HA-α'-K.d., respectively (Figure 1, Panels A and C), oligonucleotide-directed mutagenesis was used to mutate the codon for lysine to methionine at residue 68 of CK2α and at residue 69 of CK2α'. The primers used were 5'-TGGCTTGAGAATCATAACAACAACTTTTT-3' and 5'-CTGGCTTCAGGATCATTACAACCACTCT-3' for CK2α and CK2α',

respectively. The Muta-Gene[R] M13 in vitro Mutagenesis kit, Version 2 (Bio-Rad) was used for generating the mutations which were further confirmed by DNA sequencing (Sanger et al, 1977).

2.5.1.4 CK2α with C-terminal HA tag (α-Wt-HA, α-Mt-HA)

Since we do not know whether the position of epitope addition will affect the expression and functions of CK2 in cells, we created another form of CK2α by fusing the HA tag to its carboxyl terminus. To achieve this goal, a *Blp* I site was introduced at the 5' end of the HA coding sequence and a stop codon was introduced at the 3' end of the sequence encoding the HA epitope using PCR. The following primers: 5'-GCTCAGCAGTACCCATACGATGTT-3' (sense primer) and 5'-TTAAGCGTAATCTGGAACGTC-3' (antisense primer) were used. After subcloning into the TA vector, the amplified sequences were verified by DNA sequencing (Sanger et al, 1977). The TA vector encoding the modified HA sequence was digested with *Sac* I/*Blp* I and the *Sac* I/*Blp* I fragment of CK2α from the hT4.1 plasmid was subcloned into the TA vector. The HA tagged CK2α cDNA was subcloned into the *Hind* III/*Apa* I sites of pRc/CMV (Invitrogen) to generate a construct designated as α-Wt-HA (Figure 1, Panel B). The sequence of the junction region between CK2α and HA was confirmed by DNA sequencing.

To generate a construct encoding CK2α with a C-terminal HA-tag and with S/A and T/A mutations at the mitotic phosphorylation sites, α-Wt-HA was digested with *Bsu*36 I and *Blp* I. A *Bsu*36 I/*Blp* I fragment encoding the mutant form of the CTD was subcloned into the *Bsu*36 I/*Blp* I digested vector (Figure 1, Panel B). The mutant region was verified by DNA sequencing (Sanger et al, 1977). The resulting construct is designated as α-Mt-HA.

2.5.1.5 CK2α/CK2α' Chimeras

A chimera encoding the N-terminal domain of CK2α' with the C-terminal domain of CK2α designated as HA-α'/α-Wt was generated by exchanging the carboxyl domains of the respective proteins (Figure 2). *Bsu*36 I sites at nucleotide 1041 of hT 4.1 (CK2α) and at nucleotide 1058 of hT 9.1 (CK2α') were used to effect this swap. Similarly, another chimera which has the N-terminal domain of CK2α with a C-terminal domain of CK2α' named as HA-α/α'-Wt was created (Figure 2).

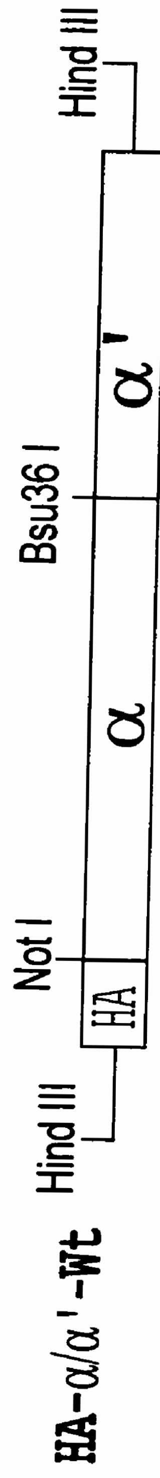
2.5.2 CK2β constructs

To permit detection of expressed CK2β in the presence of endogenous cellular CK2β, a myc epitope (MASMEQKLISEEDLNN) was ligated to the 5' end of the

Figure 2. HA tagged CK2α/CK2 α' Chimeras

Construct encoding a chimeric protein , designated HA-α'/α-Wt, with the N-terminal domain of α' and C-terminal domain of α was generated by exchanging the coding sequence of the carboxyl domains of the respective proteins. HA-α/α'-Wt was created in a plasmid encoding the N-terminal domain of CK2α with the C-terminal domain of CK2α'. Protein encoding sequences derived from CK2α are marked by the open boxes, those derived from CK2α' are marked by the shadowed boxes. The HA-α'/α-Wt and HA-α/α'-Wt constructs were subcloned into the *Hind* III site of pRc/CMV. The HA-epitope is recognized by 12CA5 monoclonal antibodies. Polyclonal anti-CK2α or anti-CK2α' antibodies recognize the carboxyl-terminal domains of the respective proteins.

HA-α'/α-Wt
Hind III
HA
Not I
α'
Bsu36 I
α
Hind III



HA-α/α'-Wt
Hind III
HA
Not I
α
Bsu36 I
α'
Hind III

coding region of CK2β in the pKS II+ vector (a gift from Dr. Stephane Richard, Washington University, St. Louis). A *Xho* I site was created to facilitate the ligation of the myc epitope sequence to the cDNAs encoding CK2β. Two forms of myc-tagged CK2β were prepared i) myc-β-Wt which encodes the entire CK2β sequence and ii)myc-βΔ1-11 which contains a deletion of its first 11 amino acids of CK2β. The latter deletion results in removal of the autophosphorylation site at Ser2 and Ser3 on CK2β. The coding region of either myc-β-Wt or myc-βΔ1-11 were obtained as *Not* I/ *Apa* I fragments that were subcloned into the respective sites of pRc/CMV (Figure 3).

To generate mutants without the mitotic phosphorylation site Ser209 on myc-β-Wt and myc-βΔ1-11, the mitotic phosphorylation site of Serine209 of CK2β was mutated to alanine by oligonucleotide-directed mutagenesis using the following mutagenic primer: 5'-GTCTTGACTGGGGCCTTGAAGTTGCT-3'. The resultant constructs were designated as myc-βSer/Ala209 and myc-βΔ1-11Ser/Ala209, respectively (Figure 3).

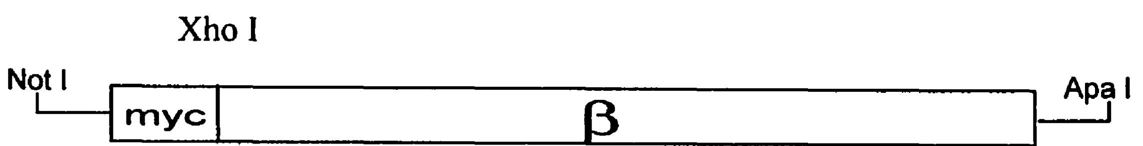
2.6 Transfection of Cells

Cos-7 cells were transfected with plasmid DNA by the calcium phosphate coprecipitation method (Ausubel et al, 1990; Graham and van der Eb, 1973). Briefly,

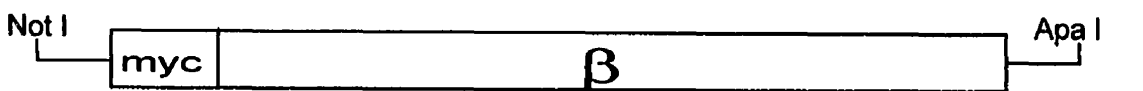
Figure 3. Myc tagged CK2β and derivatives

Constructs, designated myc-β encoding CK2β with the myc epitope at the N-terminus of the proteins were prepared as described in Materials and Methods. A *Xho* I site was used to facilitate the ligation of the myc epitope sequence to the cDNAs encoding CK2β. One mutant form of CK2β, designated myc-βΔ1-11, was generated by deletion of its first 11 amino acids including autophosphorylation sites Serine 2 and Serine 3 of CK2β. Two other mutants, designated myc-β-Ser/Ala 209, myc-βΔ1-11Ser/Ala209, were generated by mutation of the mitotic phosphorylation site, Serine 209, within C-terminal domain of CK2β into Alanine 209 on the respective proteins. Protein encoding sequences derived from CK2β are marked by the shadowed boxes, those derived from the myc epitope are marked by the open boxes. The CK2β constructs were subcloned into the *Not* I/*Apa* I sites of pRc/CMV. The myc-epitope is recognized by 9E10 monoclonal antibodies. Polyclonal anti-CK2β antibodies recognize the carboxyl-terminal domains of the respective proteins.

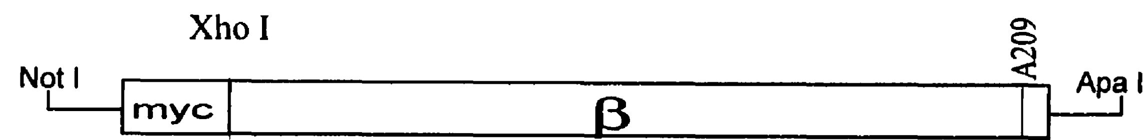
myc-β–Wt



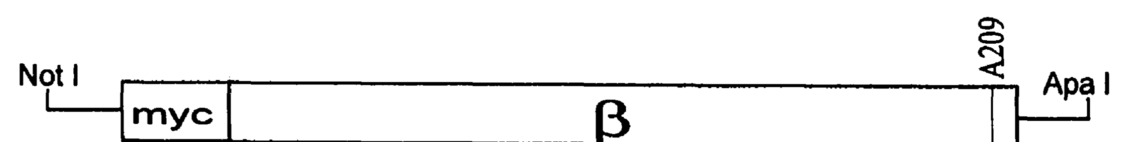
myc-βΔ1-11



myc-β–Ser/Ala209



myc-βΔ1–11Ser/Ala209



5×10^5 cells were plated in each 10 cm culture dish and incubated for 16-24 hours. The cells were refed with fresh media (10 ml/plate) and transfected with DNA mixture two to four hours later. The mixture was prepared as follows. To a sterile 14 ml tube containing 0.5 ml of 2 x HBS (0.28 M NaCl, 50 mM HEPES pH 7.08 to 7.12, 1.5 mM Na_2HPO_4), 50 µg DNA in 0.5 ml 0.25 M $CaCl_2$ was added dropwise while the 2x HeBS solution was bubbled by a mechanical pipettor attached to a sterile Pasteur pipette. The precipitates was allowed to sit 20-40 min at room temperature and then 1 ml of the DNA mixture was added dropwise to the Cos-7 cells of each 10 cm plate. After incubation of the cells with the DNA precipitate for 12-16 h, the cells were washed once with PBS , refed with fresh medium for 36-48 h and then used for experiments.

In order to monitor the transfection efficiency of Cos-7 cells, a reporter gene system of β-galactosidase in the pRc/CMV vector was created, designated as β-gal/pRc/CMV. To facilitate the cloning, a *Hind* III/*Sal* I fragment of β-galactosidase cDNA from pSV-β-gal plasmid (Promega) was ligated to *Hind* III/*Sal* I sites of pBluescript SK(+) (Stratagene), then a *Hind* III/*Apa* I fragment including the full length of β-galactosidase cDNA was cloned to the *Hind* III/*Apa* I sites of the pRc/CMV vector. To monitor the transfection efficiency, a ratio of 5 µg of the reporter gene β-gal/pRc/CMV to 40-45 µg of desired CK2 construct(s) was used for each experiment by the calcium phosphate coprecipitation. When the transfected

cells were ready for harvest, the cells were stained for β-galactosidase activity according to the method described by MacGregor et al (1991). Briefly, the cells were washed once with PBS, fixed with 0.2% glutaraldehyde for 10 min, then washed for 3 times with PBS, 5 min each. The cells were stained with X-gal solution (1 mg/ml) for 4-8 h at 37^{0}C. Transfected cells exhibited blue staining when examined under a microscope. The transfection efficiency was measured by the number of transfected cells divided by the total cells including transfected and non-transfected cells in a few random selected fields under a microscope.

2.7 Immunofluorescence

Cos-7 cells were plated onto alcohol sterilized coverslips which were placed in 6-well dishes and transfected as described above. After 48 h, the cells were washed once with PBS, fixed with 2% formaldehyde in PBS for 20 min, extracted with methanol for 20 min and then washed three times with PBS at 5 min per wash (Hall et al, 1994). The fixed cells were reacted with primary antibody in PBS containing 10 % fetal calf serum for 1 h at 37 ^{0}C and were then washed 3 times with PBS (10 min per wash). Rabbit anti-CK2α antiserum and rabbit anti-CK2α' antiserum were used at a dilution of 1/1000, and mouse monoclonal antibody 12CA5 at a 1/400 dilution. Secondary antibodies, either FITC conjugated goat anti-mouse antibody or

TRITC conjugated goat anti-rabbit antibody, were diluted 1/1000 in PBS containing 10% fetal calf serum. Reaction with secondary antibody was performed for 2 hours at room temperature in the dark and coverslips were then washed 5 times with PBS prior to mounting with 20 μl of mounting reagent (50 mg p-phenylenediamine, 5 ml 1 M Tris pH 7.4, 37.5 ml glycerol). Slides were visualized with an inverted fluorescence microscope (model Zeiss Axiovert 35 M). Control experiments were performed using peptide competition with $\alpha^{376-391}$ peptide (ANPLGMPVPAAAGAQQ) (25 μg/μl) for anti-CK2α antibodies, $\alpha'^{333-350}$ peptide (SQPCADNAVLSSGLTAAR) (25 μg/μl) for anti-CK2α' antibodies, or by using secondary antibody in the absence of primary antibody. An empty vector pRc/CMV control was also always included.

2.8 Immunoprecipitation

For immunoprecipitation, each 10 cm plate of Cos-7 cells was lysed in 1 ml lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP-40, 1% aprotinin, 0.1 mM PMSF) for 30 min on ice and the cells were scraped off the plates and transferred into microfuge tubes (Harlow and Lane, 1988). The samples were centrifuged for 10 min at 14,000 rpm in a microfuge (4 ^{0}C) and the cleared lysates were obtained. Antibodies used for immunoprecipitation were 2 μl anti-CK2α, or 2 μl anti-CK2α',

or 20 μl 12CA5 monoclonal antibody, or 2 μl anti-myc antibody (9E10) together with 30 μl of a 50% slurry of protein A Sepharose (Pharmacia). After incubation for 1 h at 4 ^{0}C, the protein A Sepharose was isolated by centrifugation and washed three times with lysis buffer. If used for electrophoresis, the samples were ready at this stage. For the kinase assay, the protein A Sepharose was washed three times with kinase buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol).

2.9 Kinase Assay

Immunocomplexes were prepared as described in the preceding section. Kinase reactions were performed in 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$, 100 μM ATP containing 0.7 μCi γ-^{32}P ATP with and without the addition of 6 μl of 1.5 mM DSD peptide substrate (RRRDDDSDDD; Litchfield et al, 1990; Kuenzel et al, 1987) in a total volume of 35 μl. The reaction was initiated by the addition of enzyme to the prewarmed buffer, and the reaction was stopped by spotting sample on Whatman P81 paper (Litchfield et al, 1990; Kuenzel et al, 1987) and washed 3 times in 0.1 % phosphoric acid, 10 min each wash. After one rinse with 95% ethanol, the filter papers were air dried and counts determined by scintillation counting. The value for the reactions lacking peptide were subtracted from the values for the reactions containing peptide.

2.10 Immunoblotting

Samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The protein was transferred to nitrocellulose or PVDF membrane for 90 min at 110 volts in blotting buffer (25 mM Tris, 190 mM glycine, 20 % methanol) (Towbin et al, 1979). The membrane was blocked with 3% gelatin in TBS (20 mM Tris-HCl pH 7.5, 500 mM sodium chloride) at room temperature for 45 min. The membrane was washed once in TTBS (0.05% Tween 20 in TBS) and incubated with either anti-CK2α (1/5000), or affinity-purified anti-CK2 α'(1/100), or 12CA5 monoclonal antibody (1 μg/ml), or 9E10 monoclonal antibody (1/500) in 1% gelatin in TTBS for 1 h at room temperature. The membrane was washed three times with TTBS. Secondary antibody was horse radish peroxidase conjugated goat-anti-rabbit or goat-anti-mouse antibody with a dilution of 1/25,000 in 1% gelatin in TTBS. The membrane was incubated with secondary antibody for 1 h at room temperature. The membrane was washed 5-8 times with TTBS. The membrane was then reacted with the enhanced chemiluminescence reagent (Amersham) for 1 min at room temperature and membranes exposed to X-ray films.

3. RESULTS

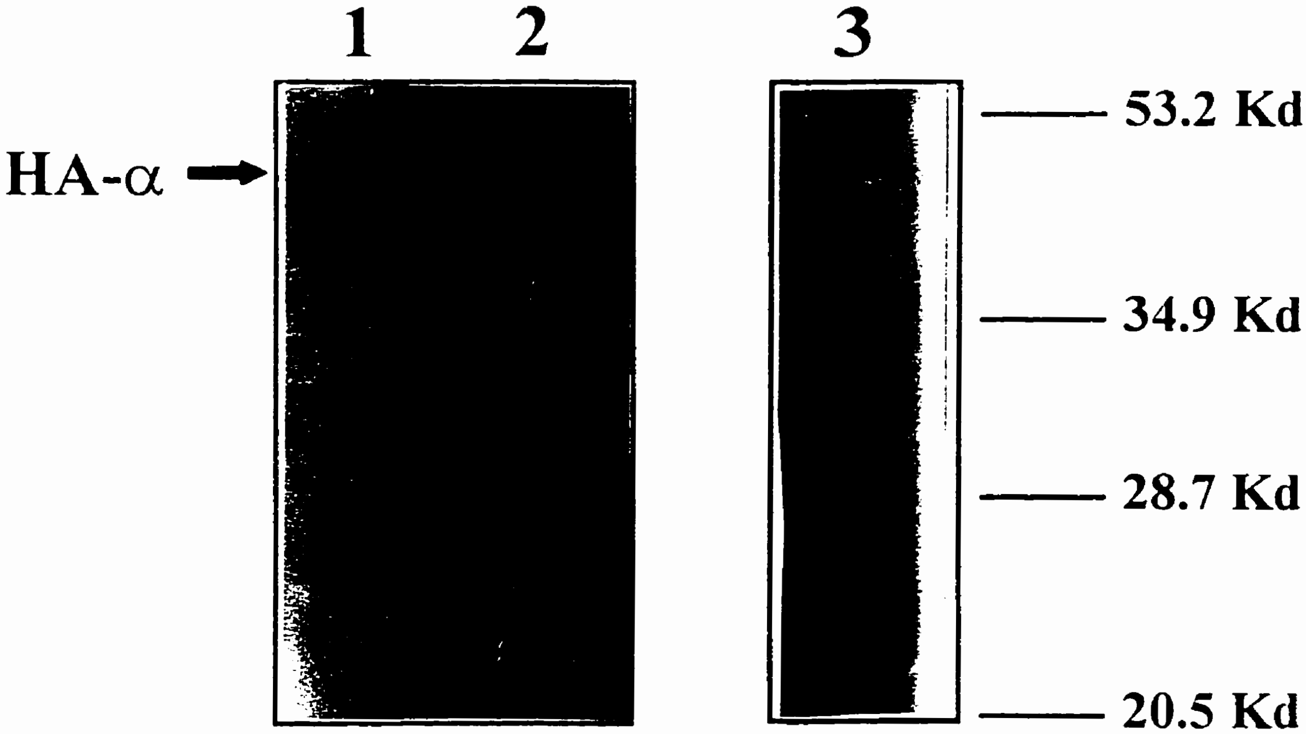
3.1 Expression of HA-α

As an initial test to determine whether the construct encoding HA-α-Wt is functional, Cos-7 cells were transfected with the pRc/CMV plasmid encoding wild type HA-α. Cell lysates were prepared, subjected to SDS-PAGE gel electrophoresis and subsequently transferred to nitrocellulose. The membrane was immunoblotted with 12CA5 antibodies which specifically recognize the HA epitope (Figure 4). Expression of HA-α-Wt (lane 1) can be detected by 12CA5 antibody, but no protein is detected in the lysate of pRc/CMV empty vector transfected cells (Figure 4, lane 3) suggesting that the 12CA5 detection is specific. This result indicated that the construct encoding HA-α-Wt, can be expressed in Cos-7 cells.

Since the ultimate objective of our study is to determine whether expression of CK2 mutants that lack its mitotic phosphorylation sites will affect cell cycle progression, a plasmid encoding the mutant form of CK2α designated as HA-α-Mt was created by mutation of the four mitotic phosphorylation sites into alanines using oligonucleotide directed mutagenesis (Bosc et al, 1995). This construct was also examined by transient transfection of Cos-7 cells. As shown in Figure 4, lane 2, the expressed protein can be detected by 12CA5 antibodies at similar levels as wild type HA-α, showing this construct can also be expressed in Cos-7 cells.

Figure 4. Expression of HA-α in Cos-7 cells

Cos-7 cells were transfected with pRc/CMV plasmids encoding HA-α-Wt (lane 1), HA-α-Mt (lane 2) or empty pRc/CMV vector (lane 3). Cell lysates were subjected to 12% SDS-PAGE gel electrophoresis and proteins transferred to a nitrocellulose membrane. Proteins were detected with 12CA5 antibody, followed by HRP conjugated goat anti-mouse antibody incubation. The results were visualized using enhanced chemiluminescence. All samples were run on the same gel. Intervening lanes with unrelated samples are not shown on this figure. The positions of molecular mass markers are indicated.


1
2
3
HA-α
53.2 Kd
34.9 Kd
28.7 Kd
20.5 Kd

To further analyze the expressed proteins, immunoprecipitations using either anti-CK2α antibodies or 12CA5 antibody were performed on extracts prepared from cells transfected with either HA-α-Wt or HA-α-Mt. Kinase activity in the immunoprecipitates was measured as described in Materials and Methods (figure 5). Figure 5A shows the results of anti-CK2α immunoprecipitates and Figure 5B shows that of 12CA5 immunoprecipitates. Kinase activity is readily detected for HA-α-Wt and HA-α-Mt in 12CA5 immunoprecipitates while minimal kinase activity is observed in pRc/CMV transfected cells. Figure 5A demonstrates there are comparable total CK2 activities for HA-α-Wt, HA-α-Mt and pRc/CMV. Collectively, these results indicate that the epitope-tagged CK2α constructs have kinase activity. However, levels of expression were not sufficient to dramatically alter total CK2 levels .

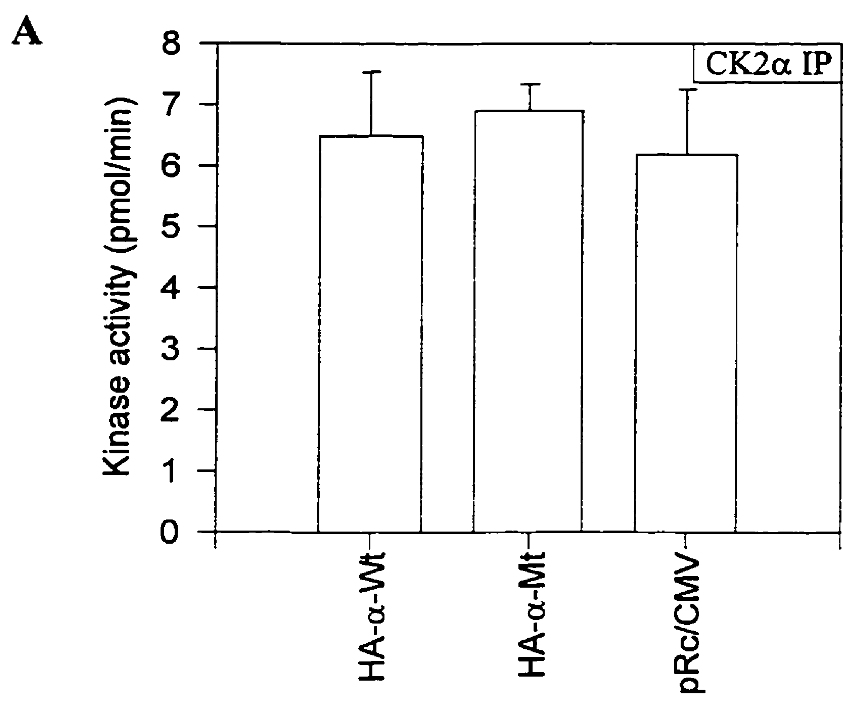
3.2 Expression of myc-β

CK2β is the regulatory subunit of CK2 and has an autophosphorylation site at Ser2 and Ser3 and a mitotic phosphorylation site at Ser209 (Litchfield et al, 1991). In order to examine the association of expressed HA tagged CK2α with CK2β subunits in transfected cells and determine the role of the phosphorylation of CK2β by other protein kinase(s) in cell cycle progression, plasmid constructs encoding

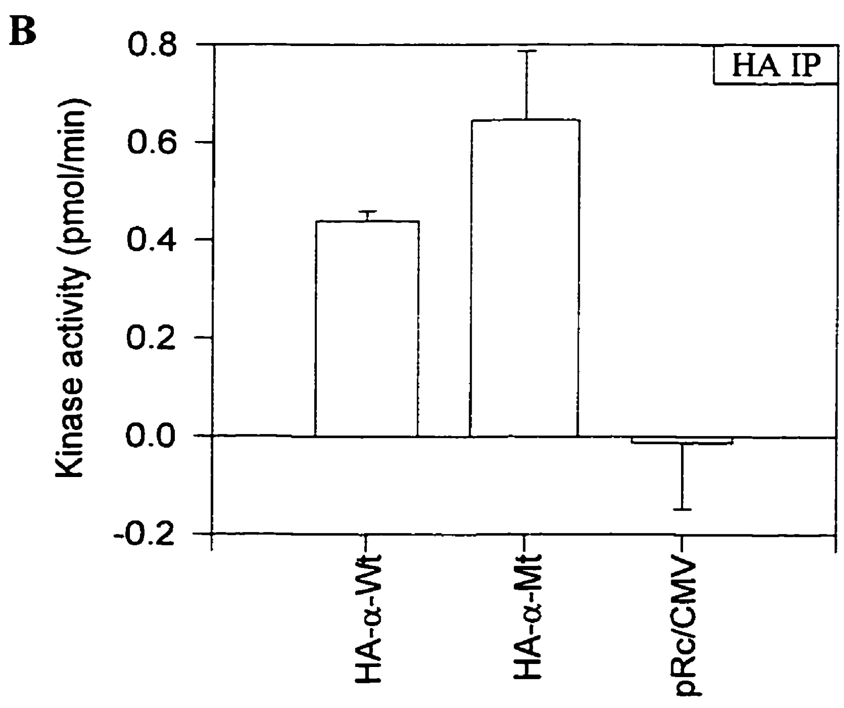
Figure 5. Kinase activity of HA-α-Wt and HA-α-Mt

Cos-7 cells were transfected with HA-α-Wt, HA-α-Mt or an empty vector pRc/CMV as a control. Cell lysates were prepared and used for immunoprecipitation with anti-CK2α antibodies (Panel A) or 12CA5 antibodies (Panel B). Immune-complex kinase assays were performed as described in Materials and Methods. Kinase activities are expressed in pmol/min. The results shown are two independent experiments and the range is indicated by error bar.

A



B



myc epitope tagged CK2β wild type and non-phosphorylatable variants were created and were tested for expression in the Cos-7 transfection system. These constructs include the myc-tagged CK2β wild type (myc-β-Wt), CK2β Ser/Ala209 mutant (myc-βSer/Ala209), Serine 2 and Serine 3 deletion mutant (myc-βΔ1-11), and a mutant with Ser 2 and Ser 3 deletion together with the Ser/Ala209 mutation (myc-βΔ1-11Ser/Ala209) (Table 1). The myc tag can be specifically recognized by 9E10 monoclonal antibodies.

Table 1 Myc tagged CK2β Wild Type and Mutants

	Serine 2	**Serine 3**	**Serine 209**
myc-β-Wt	Present	Present	Present
myc-βSer/Ala209	Present	Present	S/A Mutation
myc-βΔ1-11	Deleted	Deleted	Present
myc-βΔ1-11Ser/Ala209	Deleted	Deleted	S/A Mutation

Cos-7 cells were transfected with each of these constructs and cell lysates were prepared and analysed on immunoblots using 9E10 antibodies (Figure 6). Due to the deletion of the first 11 amino acids on myc-βΔ1-11 (lane 3) and myc-βΔ1-11Ser/Ala209 (lane 4), these bands exhibit slightly greater electrophoretic mobility than that of myc-β-Wt (lane 1) and myc-β-Ser/Ala 209 (lane 2) on the immunoblots. The protein bands are specifically recognized by 9E10 antibody since there is no

band shown in pRc/CMV empty vector (lane 5). These results indicate that the myc tagged CK2β constructs can be expressed in Cos-7 cells.

Cell lysates from transfected cells were also used for immunoprecipitation using either anti-CK2β antibodies or 9E10 antibodies. Kinase activity was measured in each of the immunoprecipitates (Figures 7). The kinase activities of 9E10 immunoprecipitates for myc-β-Wt, myc-β-Ser/Ala 209; myc-βΔ1-11, myc-βΔ1-11Ser/Ala209 (Figure 7B and 7D) are approximately 50-fold higher than that of empty vector control pRc/CMV. Since CK2β does not have kinase activity (Pinna, 1997; Cochet and Chambaz, 1983), the expressed myc-tagged β must bind endogenous α or α' subunits to form a holoenzyme which exhibit kinase activities. As was observed with HA tagged α constructs, the total CK2 activity observed was not dramatically increased by transient transfection.

The above data indicate that all of the myc tagged CK2β constructs can be expressed in Cos-7 cells and can bind catalytic CK2 subunits to form kinase active complexes. Therefore, myc-CK2β functions like endogenous CK2β and can be used to examine the subunit interaction between HA-α and CK2β in cells.

3.3 Coexpression of HA-α, HA-α' and Myc-β

The next series of experiments was performed to examine the ability of the

Figure 6. Expression of myc-CK2β and derivatives in Cos-7 cells

Cos-7 cells were transfected with pRc/CMV plasmids encoding myc-β-Wt (lane 1), myc-β-Ser/Ala209 (lane 2), myc-βΔ1-11 (lane 3), myc-βΔ1-11Ser/Ala209 (lane 4), or empty vector pRc/CMV (lane 5). Cell lysates were subjected to SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane and probed with 9E10 antibody. The results were visualized using enhanced chemiluminescence. The positions of molecular mass markers are indicated. The upper arrow points to the major bands of myc-β in lanes 1 and 2 and the lower arrow to that of myc-β in lanes 3 and 4.

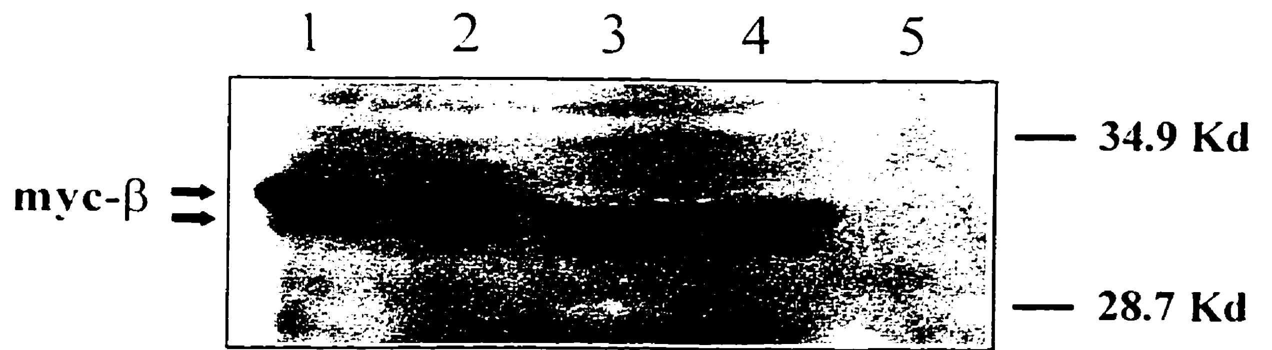
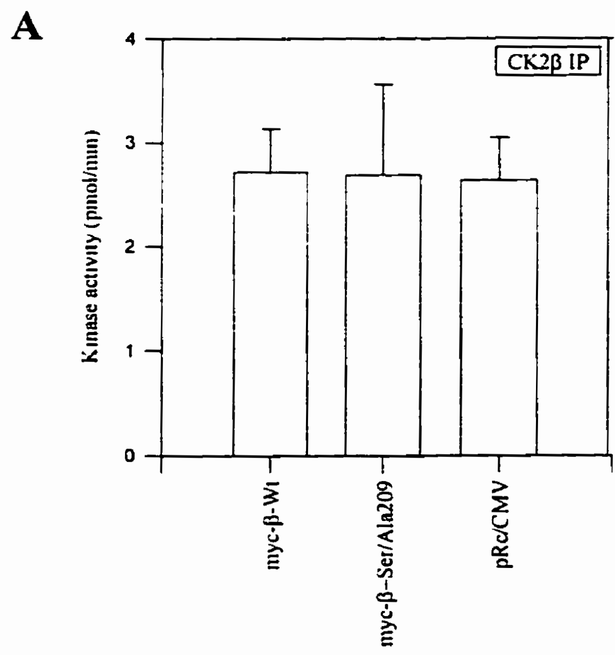

1
2
3
4
5
myc-β
34.9 Kd
28.7 Kd

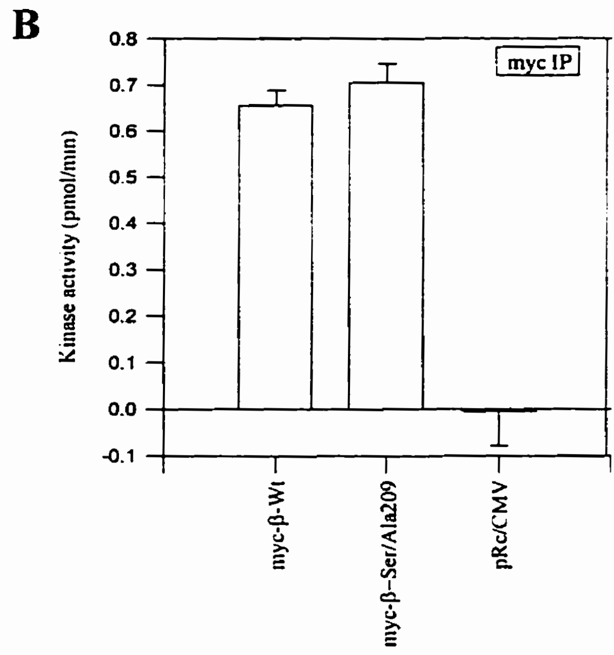
Figure 7 Kinase Activity of Myc-β and derivatives

Cell lysates were prepared from cells that had been transfected with myc-β-Wt and myc-β-Ser/Ala209 or myc-βΔ1-11 and myc-βΔ1-11Ser/Ala209 or empty vector pRc/CMV as a control. Immunoprecipitates were performed using anti-CK2β antibodies (Panels A and C) or 9E10 antibodies (Panels B and D) and immune-complex kinase assays performed as described in Materials and Methods. Kinase activities are expressed in pmol/min. The results shown are two independent experiments and the range is indicated by error bars.

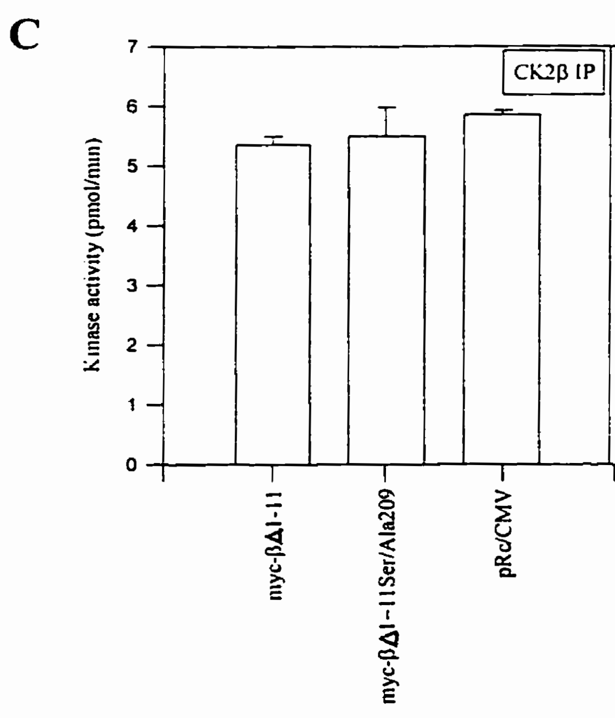
A



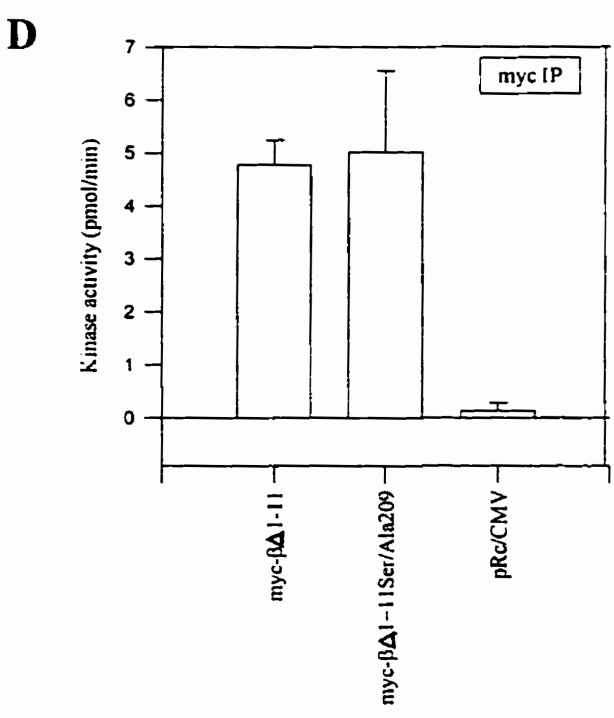
B



C



D



epitope-tagged CK2 subunits to form multi-subunit complexes. To examine complex formation, cells were transfected with combinations of HA-CK2α and myc-CK2β. Transfections were also performed with an epitope-tagged version of the other isozymic form of the CK2 catalytic subunit (ie. HA-CK2α') that was constructed by Dr. G. Penner.

Initially, the lysates from the cells co-transfected with HA-CK2α (Figure 8, lanes 1, 3, 5) and myc-β-wt or with HA-CK2α' (Figure 8, lanes 2, 4, 6) and myc-β-wt were immunoprecipitated using anti-CK2α or anti-CK2α' and immunoprecipitates were analysed on immunoblots using 12CA5 antibodies. HA-α-Wt (lane 1) and HA-α-K.d. (lane 5) were detected at much lower levels than that of HA-α'-Wt (lane 2) and HA-α'-K.d. (lane 6). The different levels of immunoprecipitated protein are not due to differences in the efficiency of immunoprecipitation since the total lysates of transfected Cos-7 also showed a similar lower expression of HA-α-Wt than HA-α'-Wt (data not shown). Since the difference between the α and α' subunits is in their divergent C-terminal domain, it is possible that the lower expression level of HA-α compared to that of HA-α' is caused by its C-terminal domain. To test this possibility, a chimeric form of the α subunit with the C-terminal domain of the α' subunit (HA-α/α'-Wt) was expressed in Cos-7 cells and the result (Figure 8, lane 3) showed that the expression level of the α/α' protein was not improved compared to HA-α-Wt (Figure 8, lane 1). In a similar vein, the expression level of a chimeric form of HA-α'

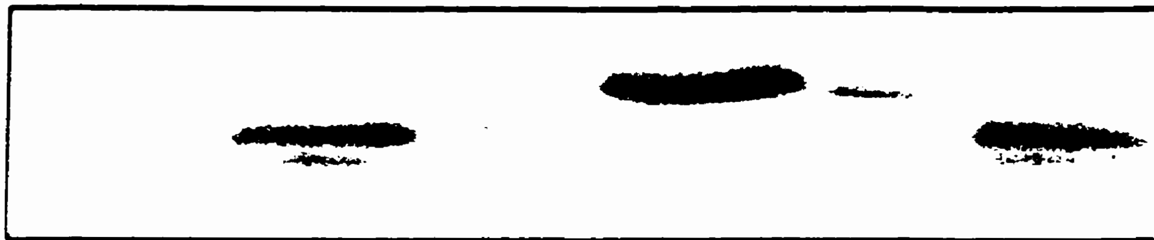
with the C-terminal domain of the α subunit (HA-α'/α-Wt) in Cos 7 cells was not adversely affected (figure 8, lane 4) compared to that of wild type HA-α' (Figure 8, lane 2). These results clearly demonstrate that the C-terminal domain of the α subunit is not responsible for the low level expression of HA-α.

For the proper function of the catalytic subunits of CK2 in cells, the catalytic subunits (α, α') must interact with the regulatory β subunits to form holoenzyme (Chester et al, 1995; Gietz et al, 1995) and the kinase activities of these catalytic subunits are generally increased upon association with the β subunits (Allende and Allende, 1995; Bidwai et al, 1993). In order to examine subunit interaction between the transfected CK2 subunits, Cos-7 cells were cotransfected with plasmids encoding HA-CK2α or HA-CK2α' and a plasmid encoding myc-β-Wt. Cell lysates were first immunoprecipitated with 12CA5 antibodies that interact with the HA epitope, and then immunoprecipitates were assayed for kinase activity (Figure 9) and on immunoblots for the presence of myc-β and for epitope-tagged CK2α or CK2α' (Figure 10).

Figure 9 shows the kinase activity of 12CA5 immune-complexes. A low level of ^{32}P-phosphate incorporation into a specific CK2 substrate was observed in immunoprecipitates from cells transfected with pRc/CMV and myc-β-Wt only (Figure 9A and 9B). Coexpression of HA-α'-Wt with myc-β-Wt resulted in a 5-fold increase of kinase activity when compared to that of HA-α'-Wt alone (Panel A) which

Figure 8. Expression of HA-α and HA-α' and derivatives in Cos-7 cells

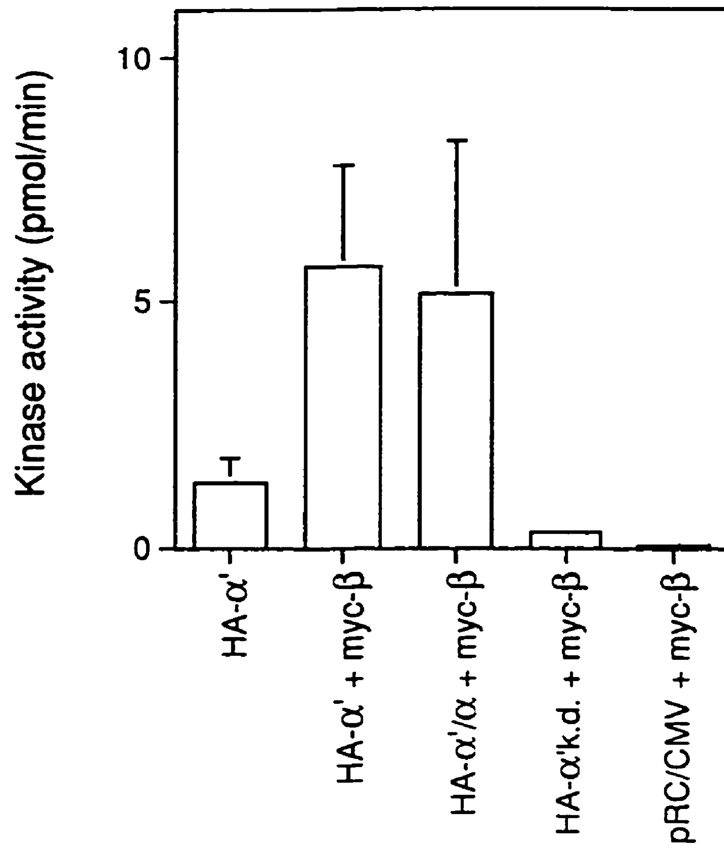
Cos-7 cells were transfected with pRc/CMV plasmids encoding HA-α-Wt (lane 1), HA-α'-Wt (lane 2), HA-α/α'-Wt (lane 3), HA-α'/α-Wt(lane 4), HA-α-K.d. (lane 5), HA-α'-K.d.(lane 6) together with myc-β -Wt. Cell lysates were immunoprecipitated with anti-CK2α (1, 4, 5) or anti-CK2α' (2, 3, 6). The immunoprecipitates were separated by 12% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane and probed with 12CA5 antibody to detect the HA-tagged proteins. Immune-complexes were visualized using enhanced chemiluminescence.

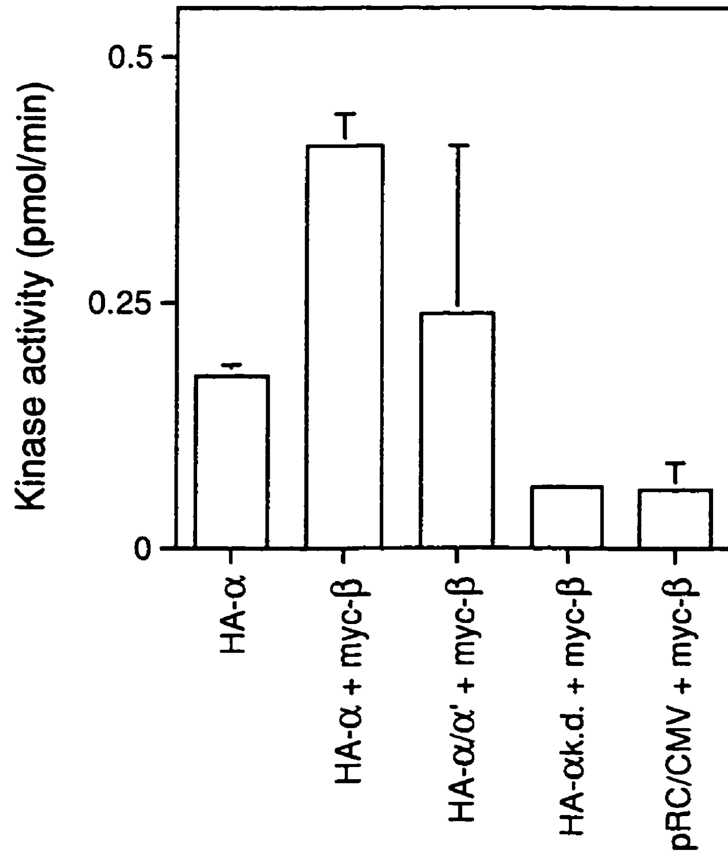

1
2
3
4
5
6

coincides with the protein level of HA-α'(-Wt) shown in Figure 10 (lanes 1 and 2) when cotransfected with myc–β-Wt, the chimeric HA-α'/α(-Wt) showed a similar kinase activity to that of HA-α'-Wt. In comparison to other HA-α'-Wt constructs, HA-α'-K.d. exhibited much less kinase activity, consist with the loss of kinase activity resulting from the K68/M mutation (Birnbaum and Glover, 1991). Since the holoenzyme protein kinase CK2 is a tetramer containing two catalytic subunits, the existence of kinase activity in 12CA5 immunoprecipitates suggest that HA-α'-K.d. may have combined with an endogenous CK2α or CK2α'. Similar results were observed with each of the HA-α constructs (Figure 9B). Similar to HA-α'-Wt, higher kinase activity is observed when HA-α-Wt is coexpressed with myc-β-Wt. Kinase activity exhibited by the chimeric constructs HA-α/α'-Wt is similar to that of HA-α-Wt while no activity above background is observed with HA-α-K.d. Overall, it is apparent that the levels of 12CA5 precipitable kinase activity present in cells transfected with HA-α'-Wt and its derivatives are significantly higher than those observed with HA-α-Wt and its derivatives (compare Figure 9A and 9B). These results are consistent with the dramatically lower levels of HA-α and its derivatives observed on immunoblots (Figure 8). Taken together, these results indicate that the kinase activities of the expressed CK2 catalytic subunits are enhanced when co-expressed with the regulatory β subunits in cells and mimic the up-regulation property of their endogenous counterparts by CK2β in cells.

Figure 9. Kinase Activity of HA-α and HA-α' and derivatives

Cell lysates were prepared from cells that had been transfected with the indicated constructs. Immunoprecipitates were performed using 12CA5 antibodies and immune-complex kinase assays performed as described in Materials and Methods. Kinase activities are expressed in pmol/min. The results shown are two independent experiments (range is indicated by error bars). Panel A. Cos-7 cells were transfected with HA-α'-Wt, HA-α'-Wt and myc-β-Wt, HA-α'-K.d. and myc-β-Wt or with pRc/CMV and myc-β. Panel B. Cos-7 cells were transfected with HA-α-Wt, HA-α-Wt and myc-β-Wt, HA-α/α'-Wt and myc-β-Wt , HA-α-K.d. and myc-β or with pRc/CMV and myc-β-Wt.


A
Kinase activity (pmol/min)
10
5
0
HA-α'
HA-α' + myc-β
HA-α'/α + myc-β
HA-α'k.d. + myc-β
pRC/CMV + myc-β

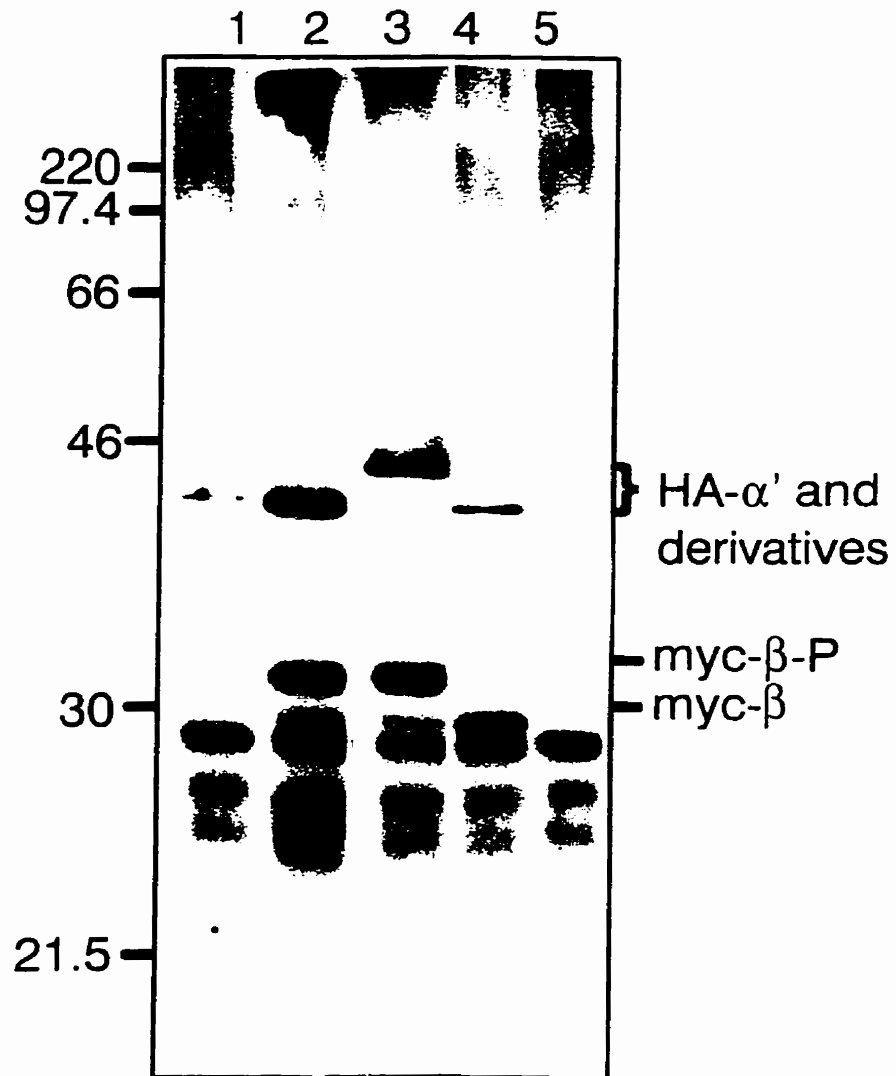

B
Kinase activity (pmol/min)
0.5
0.25
0
HA-α
HA-α + myc-β
HA-α/α' + myc-β
HA-αk.d. + myc-β
pRC/CMV + myc-β

The catalytic properties of CK2 play an important role in the functions of CK2 in cells and only the catalytic subunits of CK2 exhibit kinase activity. For proper function of CK2 in cells, the catalytic subunits must form a complex (holoenzyme) with the regulatory β subunits. To investigate the complex formation of HA-α'-Wt and its derivatives with myc-β in the cell lysates, the 12CA5 immunoprecipitates were immunoblotted using a mixture of 9E10 and 12CA5 antibodies (Figure 10). As shown in lane 2, myc-β-Wt is coprecipitated with HA-α'-Wt indicating that the HA-α'-Wt is able to associate with myc-β-Wt. This association is specific since there is no corresponding band detected in cells transfected with HA-α'-Wt alone (lane 1) or cells cotransfected with empty vector pRc/CMV and the myc-β-Wt plasmid (lane 5). In comparison to expression of HA-α'-Wt alone (lane 1), coexpression of HA-α'-Wt with myc-β-Wt elevated the expression level of HA-α'-Wt in the cells (lane 2). Also, HA-α' with C-terminal domain of CK2α (HA–α/α'-Wt, lane 3) and kinase inactive HA-α' (HA-α'-K.d. , lane 4) are also capable of forming complex with myc-β-Wt. In the latter case, the kinase inactive HA-α'-K.d. does form a complex with myc tagged β, demonstrating that the kinase activity of the catalytic subunit is not necessary for complex formation between CK2 catalytic subunits and regulatory subunits in cells. Obviously, these results suggest that the HA epitope on CK2α' and the myc epitope on CK2β do not inhibit the interaction of CK2 subunits.

Overall, these results demonstrate that HA-α or HA-α' interacts with

Figure 10. Formation of complexes between HA-α' and myc-β

Cos-7 cells were transfected with HA-α'-Wt (lane 1), HA-α'-Wt and myc-β (lane 2), HA-α'/α-Wt and myc-β (lane 3), HA-α'-K.d. and myc-β (lane 4) or pRc/CMV (empty vector) and myc-β (lane 5). Cell lysates were immunoprecipitated with 12CA5 antibody as described in Material and Methods. Immunoprecipitates were analysed on immunoblots using 12CA5 and 9E10 monoclonal antibodies to detect HA-α' or its derivatives and myc-β, respectively. Immunoreactivity was visualized by enhanced chemiluminescence. Molecular mass markers were also included in the gel and are marked. The three bands exhibiting apparent molecular weights less than that of either of the myc-β bands are immunoglobulin bands. These bands are evident in all lanes, including those derived from cells that were not transfected with myc-β.


1
2
3
4
5
220
97.4
66
46
30
21.5
HA-α' and derivatives
myc-β-P
myc-β

myc-β-Wt to form complexes (holoenzyme) in the transfected cells, and that their kinase activity and protein expression levels are enhanced upon co-expression of the β subunit. Furthermore, the kinase activity does not affect the complex formation between CK2 catalytic and regulatory subunits. Together, these results indicate the expressed HA tagged CK2α and HA tagged CK2α' function like their native counterparts in cells.

3.4 Subcellular Localization of HA-α and HA-α'

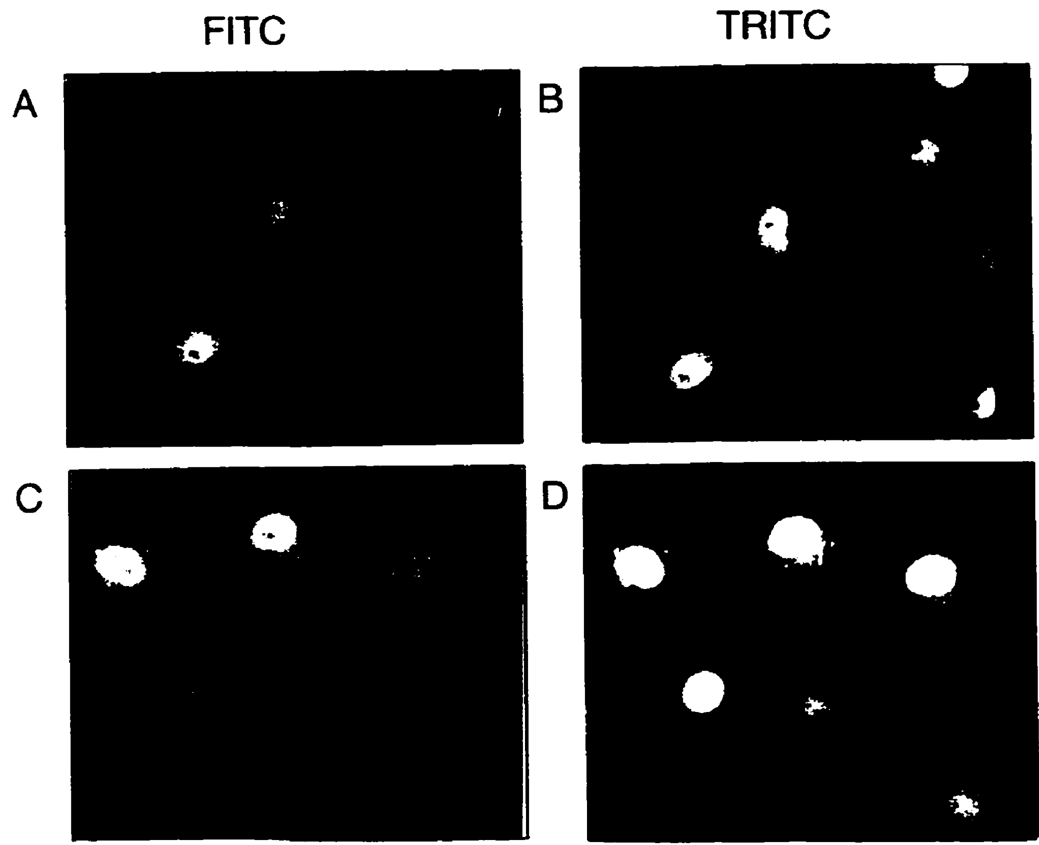
As a further test of the integrity of the epitope-tagged CK2 constructs, we were interested in examining the subcellular localization of the expressed proteins. Furthermore, we were interested in examining the subcellular localization of both CK2α and CK2α', since previous studies (Krek et al, 1992; Yu et al, 1991) failed to reach a consensus. Although Yu et al (1991) had originally observed significant differences in the subcellular localization of CK2α and CK2α', Krek et al (1992) subsequently reported that both isoforms are predominantly nuclear.

To investigate the subcellular distribution of the two isoforms of CK2, Cos-7 cells were transfected with pRc/CMV encoding HA-α-Wt or HA-α'-Wt. Expressed HA-α-Wt or HA-α'-Wt were visualized by indirect immunofluorescence using 12CA5 antibodies as the primary antibodies and FITC-conjugated anti-mouse

antibodies as the secondary antibody (Figure 11, A and C). Endogenous CK2α or CK2α' were examined on the same slides as HA-α-Wt or HA-α'-Wt using anti-CK2α antiserum or affinity purified anti-CK2α' antibodies as primary antibodies and TRITC-conjugated goat-anti-rabbit antibodies as the secondary antibodies (Figure 11, B and D). The same field was photographed with either the FITC or the TRITC fluorescence visible. Endogenous CK2α (Figure 11, B) and HA-α-Wt (Figure 11, A) are both localized primarily to the cell nucleus. Since the cells have been transiently transfected, undoubtedly the analysed cells represent a mixture of transfected and untransfected cells. For example, while a total of 6 cells are visible when staining with anti-CK2α antiserum which detects endogenous and recombinant protein, only 2 cells are visible in the same field when staining for HA-α-Wt. Endogenous CK2α' was also localized mainly to the nucleus (Figure 11, D) as was HA-α'-Wt (Figure 11, C). Most cells transfected with HA-α'-Wt also showed much brighter staining with anti-CK2α' antibodies (Figure 11, D) than did untransfected cells presumably because the overall cellular levels of CK2α' had been dramatically increased. By comparison, cells that had been transfected with HA-α did not yield such a dramatic increase in staining with anti-CK2α antisera presumably because HA-α was expressed to lower levels than HA-α'-Wt (Figure 8). In cells transfected with HA-α'-Wt and with longer exposures of staining for CK2α, CK2α' and HA-α-Wt, some cytoplasmic staining was also observed, indicating that these proteins are not

Figure 11. Indirect immunofluorescent detection of expressed HA-α and HA-α'

Cos-7 cells were transfected with pRc/CMV encoding HA-α-Wt (A, B) or with pRc/CMV encoding HA-α'-Wt (C, D). The cells were fixed and stained with 12CA5 antibodies and anti-CK2α (A, B) or with 12CA5 antibodies and anti-CK2α' (C, D) as described in Materials and Methods. The 12CA5 antibodies were detected using FITC-conjugated goat anti-mouse antibodies (A, C) while anti-CK2α (B) or anti-CK2α' (D) were detected using TRITC conjugated goat anti-rabbit antibodies. The same fields from each transfection were photographed for FITC staining and TRITC staining.

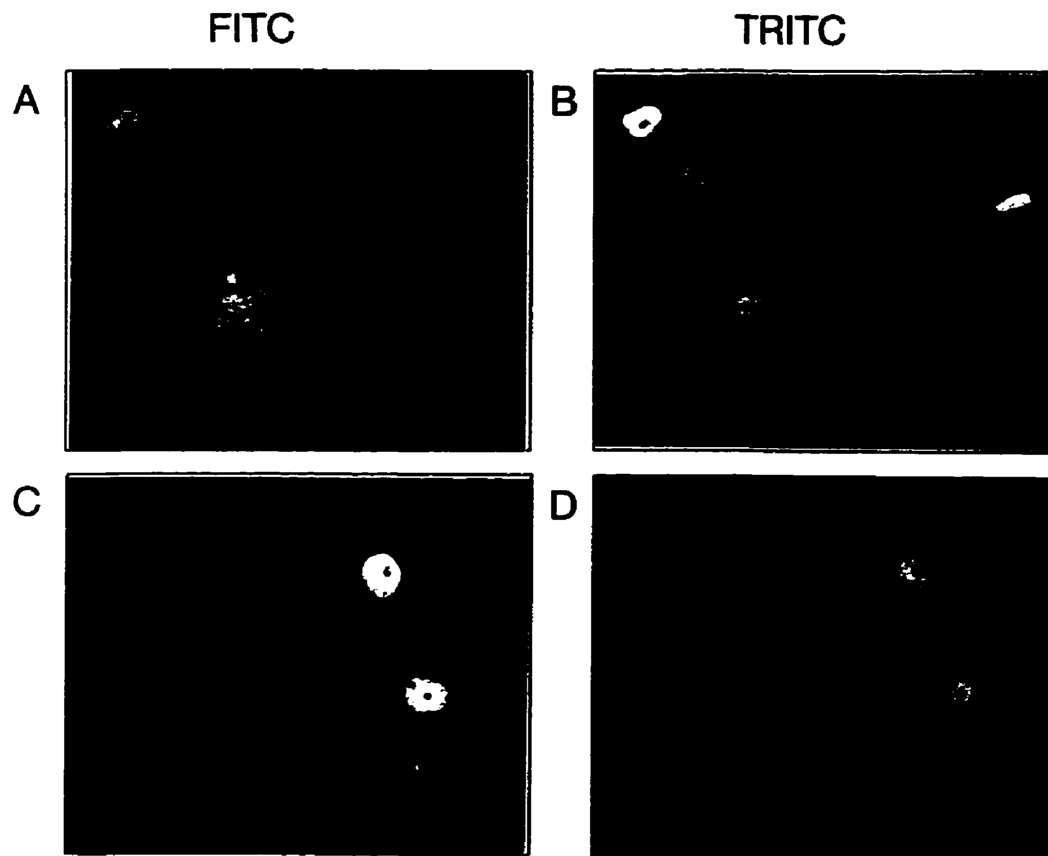

FITC
TRITC
A
B
C
D

localized exclusively to the nucleus. The similar distributions of HA-α-Wt and HA-α'-Wt with that of endogenous proteins indicates that the HA epitope does not affect the subcellular distribution of the expressed proteins.

To determine whether kinase activity is required for the appropriate subcellular localization of CK2α and CK2α', Cos-7 cells were transfected with plasmids encoding kinase inactive HA-α-K.d. or HA-α'-K.d. and analysed for the localization of expressed proteins using indirect immunofluorescent assay (Figure 12). The immunofluorescent signal from 12CA5 staining cells shows that HA-α-K.d. is predominantly localized to the nucleus (Figure 12, A). Staining of cells with anti-CK2α also reveals predominant nuclear staining as in previous experiments. Similar results are observed by examination of cells transfected with HA-α'-K.d., revealing that this recombinant protein is also localized primarily to the nucleus (Figure 12, C). As previously observed with cells transfected with HA-α', those cells transfected with HA-α'-K.d. stain much brighter than non-transfected cells when using anti-CK2α' antibodies (Figure 12, D). Once again, these results suggest that the overall levels of CK2α' have been dramatically increased in the transfected cells. Overall, the subcellular distribution of HA-α'-K.d. is very similar to that of HA-α'-Wt. These results demonstrate that catalytic function is not essential for the localization of CK2α or CK2α' to the nucleus.

Figure 12. Indirect immunofluorescent detection of HA-α-K.d. and HA-α'-K.d..

Cos-7 cells were transfected with HA-α-K.d. (A, B) or HA-CK2α' -K.d.(C, D). The cells were fixed and stained with 12CA5 antibodies (A, C) and either anti-CK2α (B) or anti-CK2α' (D) as described in Materials and Methods. The 12CA5 antibodies were detected using FITC-conjugated goat anti-mouse antibodies (A, C) while anti-CK2α (B) or anti-CK2α' (D) were detected using TRITC conjugated goat anti-rabbit antibodies. The same fields were photographed from each transfection for FITC staining and TRITC staining.


FITC
TRITC
A
B
C
D

3.5 Expression of α-HA

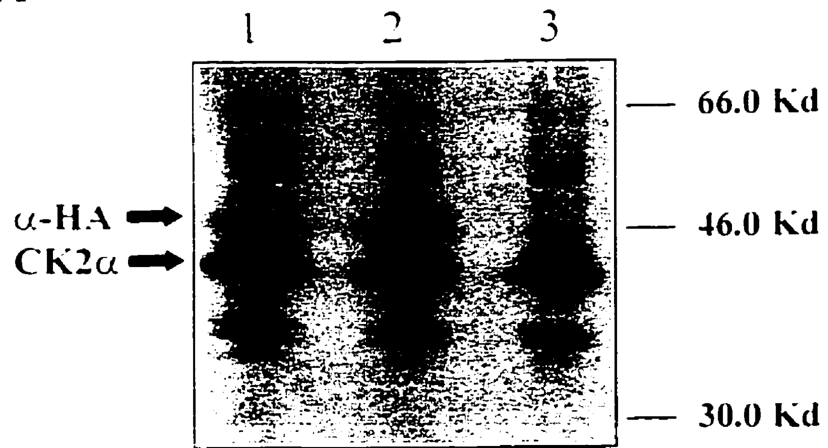
The studies performed with HA-α and HA-α' indicate that HA-α is expressed at relatively low levels. Since we do not know whether the position of the epitope addition will affect the expression and functions of CK2α in the cells, we generated another form of HA tagged CK2α by fusion of the HA epitope sequence to the C-terminus of CK2α (Figure 1, Panel B).

Cos-7 cells were transfected with pRc/CMV encoding α-Wt-HA and α-Mt-HA. Cell lysates were analysed on immunoblots using either anti-CK2α antibodies (Figure 13, Panel A) or 12CA5 antibodies (Figure 13, Panel B). In the Panel A, there are two major bands detected for α-Wt-HA (lane 1) and α-Mt-HA (lane 2), and only one major band for empty vector pRc/CMV (lane 3). Therefore, it is evident that in Panel A the lower major bands are endogenous CK2α whereas the upper major bands are expressed HA-tagged CK2α. In Panel B, the major band shown in α-Wt-HA (lane 1) and α-Mt-HA (lane 2) are expressed HA-tagged CK2α. There is also an additional upper band detected with 12CA5 antibodies in Panel B (lane 1). Since there is no upper band detected in α-Mt-HA (lane 2), the upper band shown in α-Wt-HA (lane 1) could be a phosphorylated form of HA tagged CK2α since phosphorylation of CK2α induces a shift in electrophoretic mobility (Litchfield et al, 1992). Taken together, α-Wt-HA and α-Mt-HA can be expressed in Cos-7 cells and

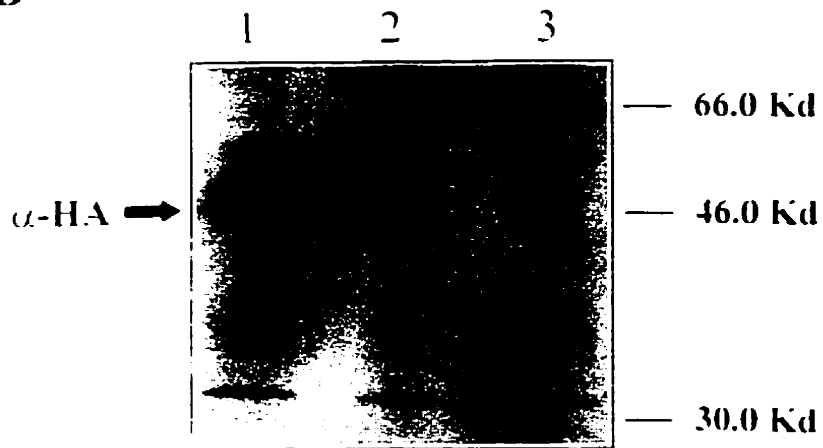
Figure 13. Expression of α-HA in Cos-7 cells

Cos-7 cells were transfected with pRc/CMV plasmids encoding α-Wt-HA (lane 1), α-Mt-HA (lane 2) or empty vector pRc/CMV (lane 3) as a control. Cell lysates were separated by 12% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membrane and probed with anti-CK2α antibody (Panel A) or 12CA5 antibody (Panel B). The results were visualized using enhanced chemiluminescence. The positions of molecular mass markers are indicated.

A



B



the expressed proteins can be detected by both anti-CK2α and 12CA5 antibodies.

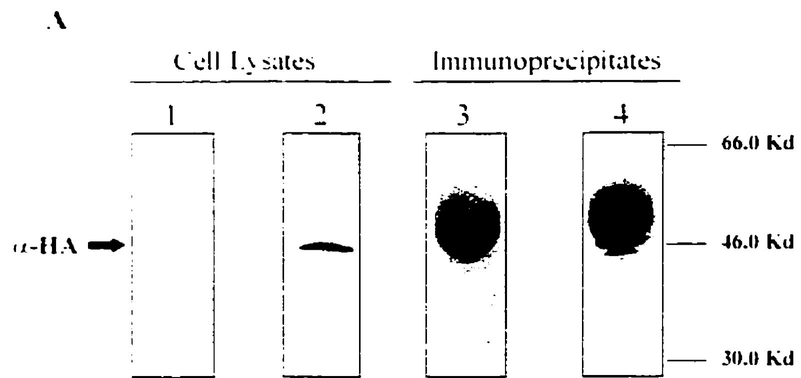
Expressed CK2α can be detected not only in cell lysates but also in immunoprecipitates as demonstrated using α-Wt-HA as an example. In Figure 14, Panel A shows that expression of α-Wt-HA can be detected using 12CA5 antibody in cell lysates (lane 2) and in immunoprecipitates performed with anti-CK2α antibodies (lane 4). However, there is no detectable signal in pRc/CMV control samples (lanes 1 and 3). In Panel B, the upper band in lane 2 is the expressed α-HA while the lower band is apparently the endogenous CK2α since it is also shown in the empty vector pRc/CMV(lane 1). The lower band in lane 4, Panel B represents α-HA since no similar band shown in pRc/CMV vector control. The major bands visible in lanes 3 and 4 of both panels are the IgG heavy chain which binds the protein A-agarose matrix during immunoprecipitation and cross-reacts with the horse radish peroxidase (HRP) conjugated anti-rabbit IgG antibody during immunoblotting. Overall, these results demonstrate that the expressed α-HA exhibits both the CK2α antigenicity and HA epitope antigenicity.

Kinase activities were measured in immunoprecipitates of cell lysates of Cos-7 cells transfected by pRc/CMV encoding α-Wt-HA and α-Mt-HA as presented in Figure 15. Panel A shows the total kinase activities for each of anti-CK2α immunoprecipitates while Panel B shows the kinase activity of expressed HA tagged proteins in 12CA5 immunoprecipitates. The expressed proteins exhibit much higher

Figure 14. Western Blotting of Expressed α-HA in Cos-7 cells

Cos-7 cells were transfected with pRc/CMV plasmids encoding α-Wt-HA (lanes 2 and 4) or empty vector pRc/CMV (lanes 1 and 3). Cell lysates were analysed on immunoblots directly using 12CA5 antibody (Panel A, lanes 1-2) or anti-CK2α antibody (Panel B, lanes 1-2). Cell lysates were also used for immunoprecipitation using anti-CK2α antibody followed by immunoblotting with 12CA5 antibody (Panel A, lanes 3-4) or using 12CA5 antibody followed by anti-CK2α antibody immunoblotting (Panel B, lanes 3-4). All samples were run on the same gel. Intervening lanes with unrelated samples are not shown on this figure. Molecular mass markers were also included in the gel and are marked. The results were visualized using enhanced chemiluminescence. Positions of endogenous CK2α and CK2α-HA are marked.

A



B

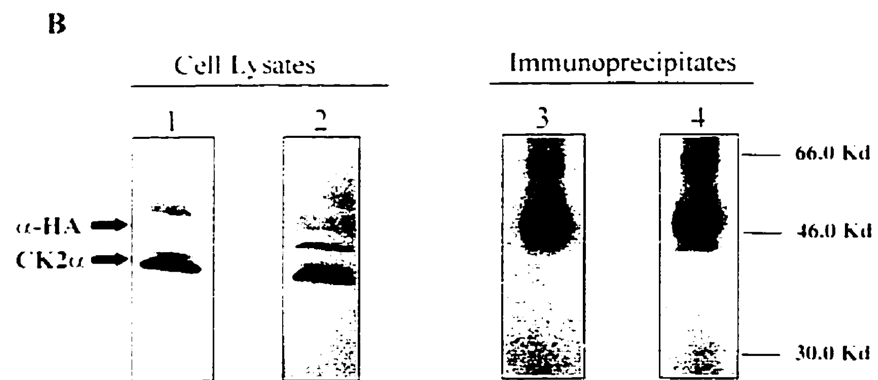
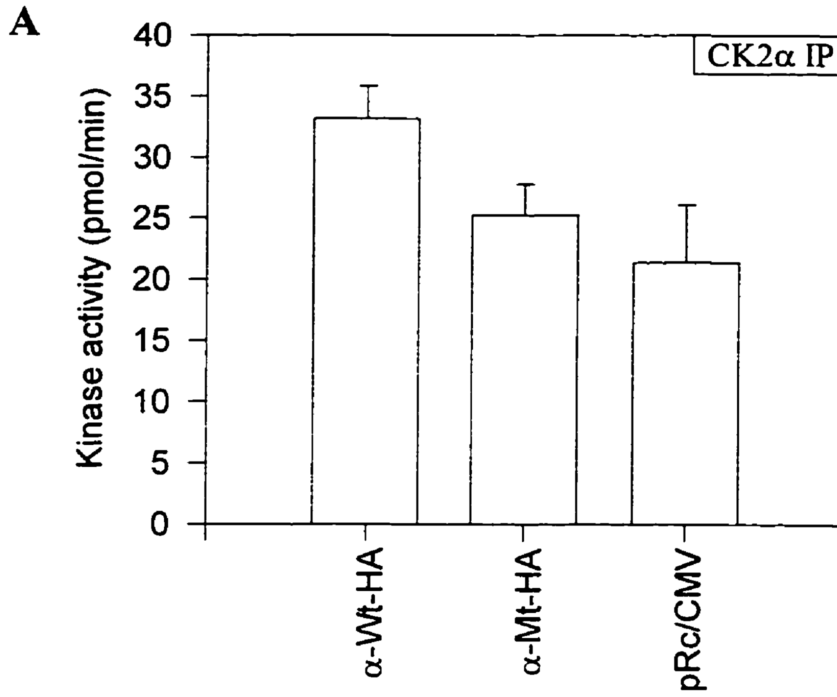
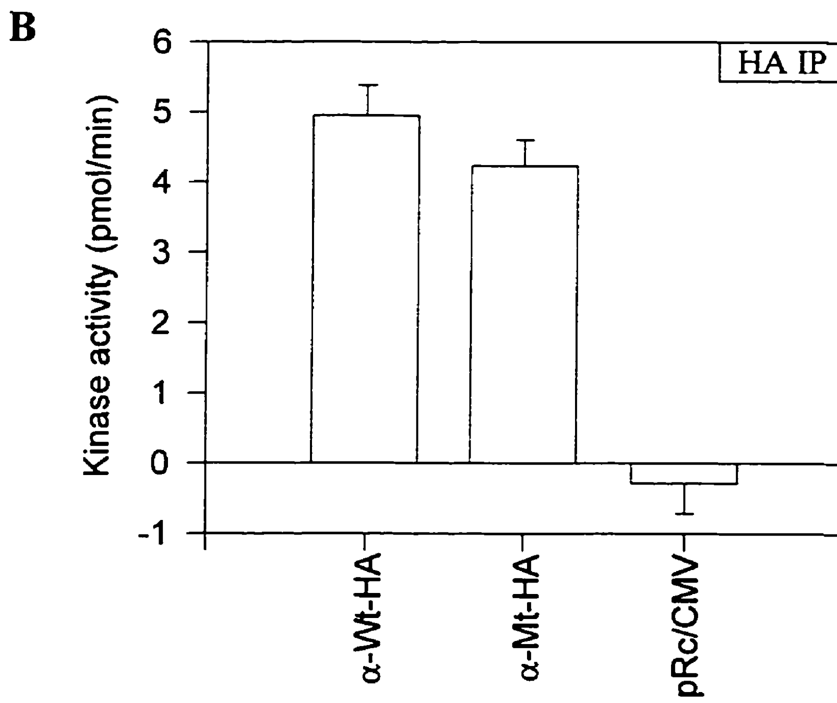


Figure 15. Kinase activity of α-HA-Wt and α-HA-Mt

Cos-7 cells were transfected with α-Wt-HA, α-Mt-HA, or pRc/CMV as a control. Cell lysates were prepared and used for immunoprecipitation with anti-CK2α antibodies (Panel A) or 12CA5 antibodies (Panel B). Immune-complex kinase assays were performed as described in Materials and Methods. The results shown are the average of two independent experiments and the ranges are indicated by error bars.


A
CK2α IP
Kinase activity (pmol/min)
40
35
30
25
20
15
10
5
0
α-Wt-HA
α-Mt-HA
pRc/CMV


B
HA IP
Kinase activity (pmol/min)
6
5
4
3
2
1
0
-1
α-Wt-HA
α-Mt-HA
pRc/CMV

activities than that of empty vector pRc/CMV, indicating HA-tagged CK2α constructs with the C-terminal HA-tag are an active kinase. Overall, the above data suggest that the expression constructs of α-HA are functional and expressed proteins retain kinase activity.

3.6 Localization of α-HA

We did not know if the position of the epitope tag could affect the localization of the recombinant protein in the cells. Cos-7 cells that had been transfected with α-Wt-HA were analysed for subcellular localization by indirect immunofluorescence. Expressed α-Wt-HA was localized primarily to the nucleus as visualized by staining with 12CA5 antibodies (Figure 16A). Endogenous CK2α in the same field was similarly localized predominantly to the nucleus when staining with anti-CK2α antibodies (Figure 16B). In the transfected cells, some cytoplasmic staining is also observed, indicating that the expressed proteins are not localized exclusively to the nucleus. The similar distribution of α-Wt-HA with that of endogenous CK2α and HA-α-Wt indicates that the HA epitope does not dramatically affect the subcellular distribution of the expressed proteins. Therefore, the position of HA epitope either at its amino terminus or at its carboxyl terminus does not affect the subcellular localization of the recombinant CK2α.

In the detection of expressed proteins in the immunofluorescent localization experiments, an empty vector pRc/CMV control is always included. For example, Cos-7 cells transfected with an empty vector pRc/CMV were immunostained with 12CA5 antibodies. No significant immunofluorescent signal is detected which suggests this 12CA5 antibody is specific to HA epitope (Figure 16C). Furthermore, endogenous CK2α is visualized by staining with anti-CK2α antibodies (Figure 16D).

Besides the pRc/CMV control in every transfection experiment, a number of other immunostaining controls are always included. Peptide competition using $\alpha^{379\text{-}391}$ peptide for anti-CK2α antibodies or $\alpha'^{333\text{-}350}$ peptide for anti-CK2α' antibodies completely block out the signal detected by the respective anti-CK2α or anti-CK2α' antibodies (data not shown). Also, the use of secondary antibodies without previous incubation with primary antibodies does not give any detectable FITC signal and TRITC signal (data not shown). Taken together, these results indicate that 12CA5 antibody, anti-CK2α and anti-CK2α' antibodies are specific to their own epitope, and there is no significant non-specific staining in the immunofluorescent assay experiments.

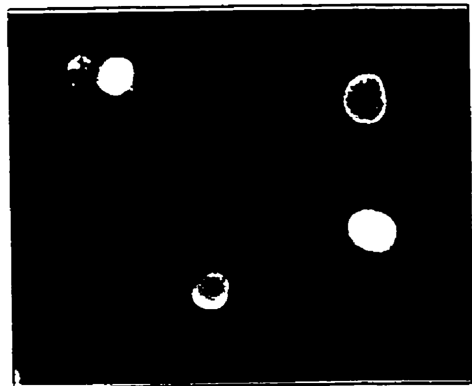
Furthermore, in order to monitor transfection efficiency for each plasmid in every transfection experiment, Cos-7 cells were always co-transfected with a desired plasmid of CK2 and β-gal/pRc/CMV. Histochemical assay of β-galactosidase

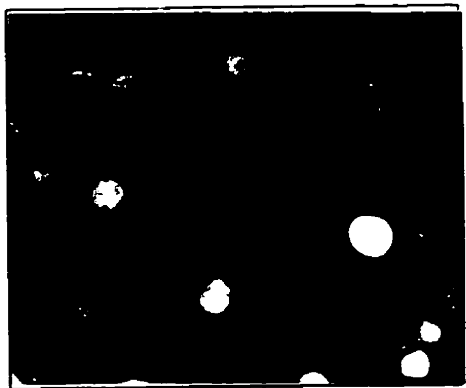
activity by X-gal staining was carried out and the percentage of blue stained cells verus total cells indicated the efficiency of transfection. The amount of each plasmid DNA encoding CK2 subunit was adjusted for each transfection experiment in order to achieve a similar transfection efficiency among plasmids used (data not shown).

Figure 16. Indirect immunofluorescent detection of α-Wt-HA and pRc/CMV

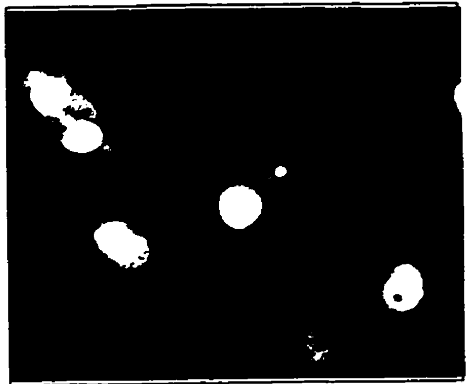
Cos-7 cells were transfected with α-Wt-HA (Panels A and B) and empty vector pRc/CMV as a control (Panels C and D) . The cells were fixed and stained with 12CA5 antibodies and anti-CK2α antibodies as described in Materials and Methods. The 12CA5 antibodies were detected using FITC-conjugated goat anti-mouse antibodies (A, C) while anti-CK2α (B, D) were detected using TRITC conjugated goat anti-rabbit antibodies. The same fields were photographed from each transfection for FITC staining and TRITC staining.

FITC
TRITC

A


B


C

D


4. DISCUSSION

Several lines of evidence suggest that CK2 is an important component of signalling pathways with functions that are critical at a number points of the cell cycle. At the early stage of the cell cycle, increases in the synthesis of CK2 and in its translocation to the nucleus are required for cell cycle progression (Allende and Allende, 1995). In avian and mammalian cells at mitosis, CK2α is phosphorylated to high stoichiometry likely by $p34^{cdc2}$. It is proposed that this cell cycle dependent phosphorylation could alter the functional properties of CK2 and further regulate cell division (Litchfield et al, 1992). Recently, the mitotic phosphorylation sites on CK2α have been identified as Threonine344, Threonine360, Serine362 and Serine370 which are located to the unique carboxyl terminal domain of CK2α (Bosc et al, 1995). Consequently, one approach for examining the effects of CK2 and its phosphorylation on cell cycle progression could be to examine the effect of expressing forms of CK2 that harbor mutations at the mitotic phosphorylation sites.

In order to determine whether the mitotic phosphorylation of CK2 is essential for cell cycle progression, I initially created different constructs encoding non-phosphorylatable forms of CK2 and tested whether these constructs are functional by transient transfection of Cos-7 cells. Second, in order to investigate whether the translocation of expressed non-phosphorylatable forms of CK2 into the nucleus is affected by the mutations, I examined the feasibility of using epitope-tagged CK2 constructs to investigate the subcellular localization of CK2 in cells by

immunofluorescent microscopy.

4.1 Creation of CK2 constructs and expression of the CK2 plasmids in Cos-7 cells

When we initiated these studies the only report on the over-expression of CK2 in mammalian cells was from Heller-Harrison et al (1991) who demonstrated increases in total CK2 protein and kinase activity in Cos-1 cells transfected with either CK2α subunit or CK2β subunit or co-transfected with the both CK2α and CK2β subunits. Since those results were based on the analysis of total cell lysates of transfected Cos-1 cells, in which there are endogenous CK2 subunits and expressed exogenous counterparts, it is not possible to define the exact contribution of the expressed exogenous CK2 subunits to the elevation of the protein level and kinase activity in the Cos-1 cells. Therefore, it is important to distinguish exogenous CK2 from their endogenous counterparts in transfected cells. Furthermore, the studies of Heller-Harrison et al (1991) were restricted to an analysis of wild-type CK2α and CK2β.

In order to distinguish expressed CK2 subunits from their endogenous counterparts in cells, an epitope was fused to the N-terminus or C-terminus of CK2α and to the N-terminus of CK2β. The HA and Myc epitopes used in these studies are

readily detected with commercial monoclonal antibodies. There are several criteria to ensure that there is no effect on the functions of the tagged protein as compared to the native protein. Specifically for CK2, the addition of epitope tag should not abolish the catalytic activity of tagged CK2α, it should not affect the association between CK2α and CK2β, nor should it affect the subcellular localization of CK2 in cells.

In order to ultimately investigate the role of mitotic phosphorylation sites of CK2 during cell cycle progression, non-phosphorylatable forms of CK2 were created. To initially test whether these plasmid constructs are functional and whether the epitope addition has any effect on the properties of expressed CK2 subunits, transfection studies were conducted in Cos-7 cells. Results from Western blotting and immune-complex kinase assays show that HA-α-Wt and HA-α-Mt can be expressed in Cos-7 cells and display kinase activity (Figures 4 and 5). These results suggest that these constructs were functional and that the HA epitope on CK2 did not affect their catalytic properties nor affect their antigenicities. For testing the effect of the epitope tag on the association of CK2α or CK2α' with the regulatory CK2β subunits in transfected cells, co-expression of HA-α-Wt, HA-α'-Wt with myc-β-wt were carried out in Cos-7 cells (Figures 9 and 10). Kinase activities and co-immunoprecipitation assays indicate that complexes between HA-tagged catalytic subunits and myc-tagged regulatory subunits are formed confirming that the epitope

tags do not interfere with CK2 holoenzyme assembly.

Kinase activity is an important property of CK2 since the functions of CK2 are believed to be mediated by its ability to phosphorylate its respective substrate proteins. To examine the importance of catalytic abilities of CK2, kinase inactive variants of CK2α (HA-α-K.d.) and CK2α' (HA-α'-K.d.) were created in expression plasmids by mutation of one of their residues (K/M 68 (α) or K/M 69 (α')) within their ATP binding domain known to be crucial for catalytic activity in other protein kinases (Birnbaum and Glover, 1991; Hanks et al, 1988) and were expressed in Cos-7 cells. The results in Figure 10 indicate the expressed HA-α-K.d and HA-α'-K.d. can be immunoprecipitated by anti-CK2α and anti-CK2α' antibodies, respectively, and the immunoprecipitated proteins can be detected by anti-HA epitope antibody 12CA5, suggesting the constructs are functional and expressed in Cos-7 (Figure 8). Kinase activity analysis (Figure 9) shows these expressed proteins did not exhibit catalytic activities which is in agreement with their K/M 68 (α) or K/M 69 (α') mutation as described by Birnbaum and Glover (1991). These results further confirm that inactivity of CK2 catalytic subunits is directly caused by these mutations (K/M 68 (α) or K/M 69 (α')) since these residues play a critical role in catalysis (Hanks et al, 1988). Furthermore, co-expression of kinase inactive HA-α' with myc tagged CK2β indicated that HA-α'-K.d. directly interact with myc tagged CK2β (Figure 10, lane 4), demonstrating that the kinase activity is not required for

CK2 holoenzyme formation in cells. These mutants can be used to study the role of catalytic activity of CK2 during cell cycle progression.

Since we did not know whether the position of epitope addition on CK2 has any effect on the expression of CK2, and the amino-terminal tagged CK2α (HA-α-WT) exhibits low expression level of protein (Figures 4 and 5), another form of HA tagged CK2α (α-Wt-HA) was created with the HA epitope at its C-terminus and tested in Cos-7 cells. A non-phosphorylatable form (α-Mt-HA) was also constructed in expression plasmids. Expression in Cos-7 cells indicated the plasmids are functional on the basis that the expressed proteins display kinase activity and that C-terminal tagged CK2α exhibits nuclear localization (Figures 15 and 16). The position of HA tag seems to favour the expression of this form of HA tagged CK2 because the expressed proteins can be easily detected by 12CA5 antibody (Figure 13). In the absence of a direct comparison between the HA-CK2α and CK2α-HA constructs, it can not be definitely concluded that the c-terminal tag results in a higher level of expression. However, our results are consistent with this conclusion. It will therefore be of interest to test this possibility in the future.

Since the expression level of HA-CK2α was much lower than that of HA-CK2α' (Figure 8) and the major difference between these two proteins is their divergent C-terminal domains (CTD) (Lozeman et al, 1990), we thought that the low level of expression of CK2α might be cause by its CTD. To test this possibility, a

chimeric form of CK2 named HA–α'/α-Wt which has the amino terminal domain of CK2α' with the CTD of CK2α was created and tested for its expression in Cos-7 cells. The results reveal that the expression of this protein was similar to that of CK2α' (Figure 8), indicating that the low level of expression of CK2α is not directly caused by its CTD. Moreover, it is unlikely that a random mutation of the parent pRc/CMV plasmid is responsible since substituting the α' sequence for the α sequence in HA-α did not result in decreased expression of HA-α' protein (data no shown). Whether this low level expression of HA-α constructs is due to a possible lethal effect of expression remains to be determined.

In this study, we observed an enhancement of kinase activity and CK2 protein when CK2α or CK2α' were co-expressed with CK2β. This result is consistent with the results from the transfection studies of Heller-Harrison et al (1991). In a similar vein, Filhol et al (1991) also observed optimal expression of CK2 in insect cells when CK2α and CK2β are co-expressed. These results suggest that CK2β has a role in stabilizing the CK2α protein.

The β subunits are the regulatory subunits of CK2 which are involved in enhancing the catalytic activity of the enzyme and regulating its specific interactions with some substrates and inhibitors (Allende and Allende, 1995). CK2β is phosphorylated at its autophosphorylation sites of serine 2 and serine 3 apparently at all stages of the cell cycle and at its mitotic phosphorylation site (serine 209) at

mitosis (Boldyreff et al, 1993; Litchfield et al, 1991; Litchfield et al, 1992). Using *in vitro* reconstitution assays with purified proteins, mutation and deletion of these phosphorylation sites did not prevent the association of CK2α and CK2β and the activation of CK2α by the β subunits (Hinrichs et al, 1993; Meggio et al, 1993; Palen and Traugh, 1991). Importantly, serine 209 of CK2β is conserved among species such as human (Jakobi et al, 1989; Heller-Harrison et al, 1989), murine (Kopatz et al, 1990), bovine (Takio et al, 1987) and chicken (Maridor et al, 1991) indicating this residue may play an important role in the regulation of functional properties of mammalian or avian CK2. In order to investigate the possible role of these phosphorylation sites during cell cycle progression, we created plasmid constructs encoding the wild type and phosphorylation mutants of CK2β and introduced them into Cos-7 cells. An epitope of human c-myc was fused in frame to the amino terminus of the CK2β to distinguish them from the endogenous cellular CK2β subunits. Expression of myc tagged CK2β and its phosphorylation mutants were also carried out in Cos-7 cells. The results indicate that these constructs can be expressed in Cos-7 cells and the expressed CK2β can be associated with CK2 catalytic subunits to form kinase active complexes. The latter instance has been further demonstrated with wild-type CK2β that when coexpression with HA-CK2α' in the cells, myc-β can be co-immunoprecipitated with expressed CK2α' providing evidence of the physical association of myc-β with the α' catalytic subunits.

Furthermore myc-β mutants with deletion of the first 11 amino acid residues including the autophosphorylation sites did not prevent association with CK2α subunits as indicated by the existence of kinase activity in anti-myc immunoprecipitates, suggesting that the region of amino acid residues 1-11 of CK2β is not directly responsible for the interaction between subunits.

4.2 Localization of HA-α , HA-α' and α-HA

Studies have shown that CK2 can phosphorylate more than 120 different proteins which are located within the nucleus and the cytoplasm of cells (Pinna, 1997; Pinna et al, 1994). The distribution of these substrate proteins suggests that CK2 may be located in different cellular compartments. Recent studies have confirmed that CK2 is localized within the nucleus and the cytoplasm (Filhol et al, 1990). It is suggested that the nuclear/cytoplasmic distribution of CK2 is quite different between proliferative cells and quiescent cells. CK2 is more prominently localized to the nucleus in proliferative cells (Filhol et al, 1990). Importantly, through the phosphorylation of the regulatory nuclear proteins such as transcriptional factors, CK2 could regulate their functions and play an essential role in the regulatory pathway controlling proliferative events (Litchfield and Luscher, 1993).

Two distinct isoforms of the catalytic subunits of CK2, CK2α and CK2α' have

been identified in yeast, mammals and birds (Chen-Wu et al, 1988; Padmanabha et al, 1990; Meisner et al, 1989; Lozeman et al, 1990; Maridor et al, 1991; Takio et al, 1987; Lozeman et al, 1990; Litchfield et al, 1990). Previous studies from different laboratories arrived at significantly different conclusions regarding the subcellular location of endogenous CK2α and CK2α'. Yu et al (1991) reported dramatic differences in the subcellular localization of CK2α and CK2α'. In interphase cells, the authors concluded that CK2α was primarily located within the cytoplasm and CK2α' predominantly to the nucleus. In contrast, a subsequent study by Krek et al (1992) presented evidence of very similar subcellular distribution of these two isoforms, predominantly nuclear localization. There are limitations regarding conclusions drawn from studies performed with endogenous CK2. Given the close similarity between CK2α and CK2α', many antibodies against CK2 lack isozyme specificity. In fact, the conclusions of Yu et al (1991) were in part based on analysis using antibodies that display reactivity with both isoforms. In addition, CK2 is known to exist in homotetrameric ($\alpha_2\beta_2$, $\alpha'_2\beta_2$) and heterotetrameric ($\alpha\alpha'\beta_2$) complexes (Chester et al, 1995; Gietz et al, 1995). Therefore, unless the composition of these complexes is known, it is difficult to draw definitive conclusions regarding the localization of CK2α and CK2α'. To circumvent these problems and to resolve discrepancies regarding the subcellular localization of CK2α and CK2α', in this study the subcellular distribution of epitope-tagged variants of the two isoforms was

examined. The HA-epitope facilitates detection of expressed proteins above a background of endogenous CK2 without concerns of cross-reactivity with other CK2 isoforms. Furthermore, since transfected cells overexpress only one isoform which can be specifically detected by the anti-epitope antibodies, our results are not confounded by the existence of tetrameric complexes containing both CK2α and CK2α'. Overall, our results support the conclusion that both CK2α and CK2α' are predominantly localized to the nucleus.

The subcellular localization of the kinase inactive variants of HA-α and HA-α' suggests that kinase activity is not required for localization of CK2 to the nucleus. At present the factor(s) that controls the transport of CK2 to the nucleus remains unclear. Since both CK2α and CK2α' contain sequences that conform to known nuclear localization signals, the ability of both isoforms to localize to the nucleus may residue within these sequences. However, since CK2α and CK2α' both form complexes with CK2β, it is possible that CK2β plays a role in guiding the subcellular localization of CK2.

Sequence comparison indicates that the α and α' subunits are very similar within the amino termini. By comparison, the carboxyl termini of the α (61 amino acids) and α' (21 amino acids) are completely unrelated (Lozeman et al, 1990; Maridor et al, 1991). Although the existence of remarkable conservation of these two isoforms reveals that they may have overlapping functions, their divergent C-terminal

domains suggest that they may have some unique functions or that they may be regulated by different mechanisms. Particularly, only the α (not α') subunit has been identified to have mitotic phosphorylation sites which locate to its unique carboxyl terminal domain and has been demonstrated to be dramatically phosphorylated at mitosis (Litchfield et al, 1992; Bosc et al, 1995). Therefore, the mitotic phosphorylation of CK2α by other protein kinases, $p34^{cdc2}$ for example, could be one important mechanism to regulate the function of CK2α during cell cycle progression. Our observation that CK2α is primarily localized within the nucleus supports a number of reports that the synthesis of CK2 and its translocation into the nucleus are required for cell proliferation (Krek et al, 1992; Filhol et al, 1990; Gauthier Rouviere et al, 1991; Belenguer et al, 1989; Lorenz et al, 1993; Pyerin et al, 1992; Pepperkok et al, 1991).

5. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, from the data presented above, the expression constructs encoding wild type and mutant forms of CK2α, CK2α' or CK2β have been created and shown to be functional in the Cos-7 expression system. The epitope addition affects neither the catalytic properties of tagged catalytic subunits (α, α') nor the interactions between the catalytic subunits and the regulatory subunits (β) in cells. The expressed HA tagged CK2α and CK2α' exhibit kinase activity and can be detected by anti-HA epitope antibodies and by respective antibodies against them. Co-expression of the HA tagged catalytic subunits with myc tagged CK2β in Cos-7 cells demonstrates that the expressed catalytic subunits do interact with CK2β subunits and therefore exhibit enhanced kinase activity and physical association with myc tagged CK2β shown in immunoblots after immunoprecipitation. Furthermore, a small region of residues 1-11 including the autophosphorylation sites on CK2β is not essential for the interactions between the subunits of CK2. Subcellular localization studies have illustrated the expressed HA tagged CK2α and CK2α' primarily localize to the nucleus of cells behaving like their endogenous counterparts. Additionally, the localization of kinase inactive HA-α-K.d. and HA-α'-K.d. primarily to the nucleus of cells demonstrates that the kinase activity of CK2 is not required for the subcellular localization.

These studies lay the groundwork for a systematic examination of CK2 and its phosphorylation during cell progression. One approach for examining the role of CK2

during cell cycle progression is to co-transfect a plasmid encoding a cell surface marker such as CD20 to facilitate identification of transfected cells (van den Heuvel and Harlow, 1993). Alternatively, it may be more suitable to use an inducible expression system because the expression level of CK2 can be manipulated or limited to a certain extent to prevent an overexpression of CK2 (Gossen and Bujard, 1992; Resnitzky et al, 1994).

The choice of cell line for cell cycle distribution study may be important for CK2. On preliminary examination, CK2 mutants expressed in Cos-7 cell did not produce obvious changes in cell cycle distribution (data not shown). One possible explanation is that Cos-7 cells might not be a suitable host because they constitutively produce SV40 large T antigen which will have an effect on cellular p53 protein that perturbs normal cell cycle regulation (May and May, 1995; Quartin et al, 1994; Ludlow, 1993). Successful cell cycle distribution studies have used Saos-2 or U-2 OS or C33A cell lines (van der Heuvel and Harlow, 1993) and these cell lines may be good candidates for the study of the effects of CK2 on the cell cycle.

Overall, the studies described in this thesis clearly demonstrate all the CK2 plasmids created are expressed in Cos-7 cells and expressed proteins behave in a similar manner to their endogenous counterparts. These studies build a solid foundation for further investigation of the role of mitotic phosphorylation of CK2 during cell cycle progression.

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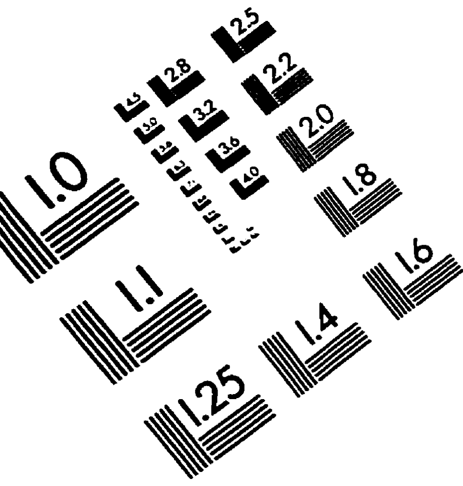
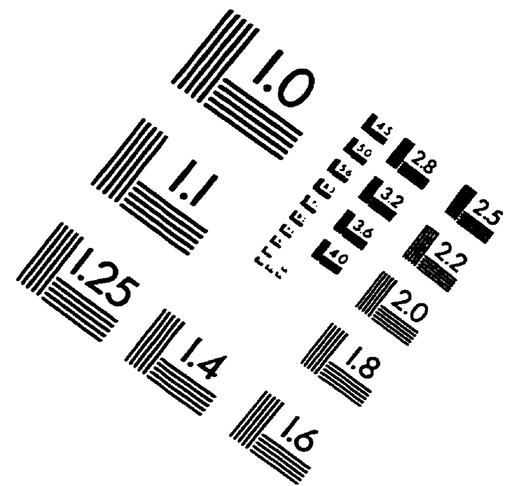
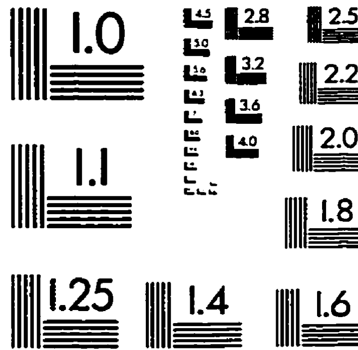
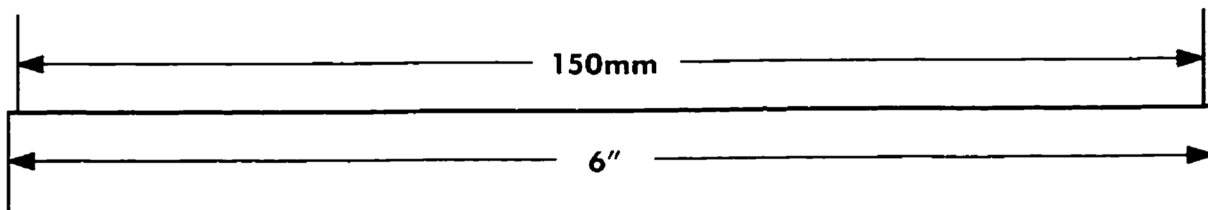
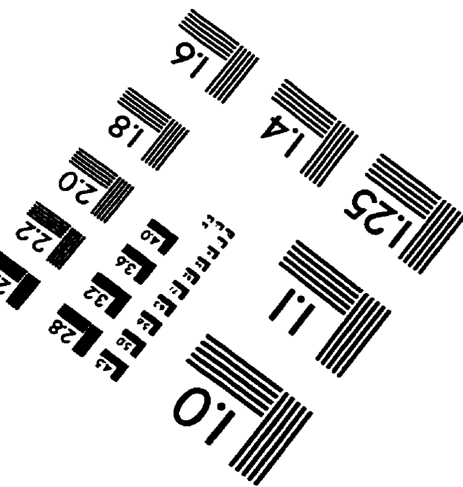
IMAGE EVALUATION TEST TARGET (QA-3)











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