Expression of the Casein Kinase 2 Subunits in Chinese Hamster Ovary and 3T3 L1 Cells Provides Information on the Role of the Enzyme in Cell Proliferation and the Cell Cycle*

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In order to investigate the in vivo functions of protein kinase CK2 (CK2), the expression of Myc-tagged versions of the subunits, Myc-CK2α and Myc-CK2β, was carried out in Chinese hamster ovary cells (CHO cells) and in 3T3 L1 fibroblasts. Cell proliferation in these cells was examined. CHO cells that transiently overexpressed the Myc-CK2β subunit exhibited a severe growth defect, as shown by a much lower value of [3H]thymidine incorporation than the vector controls, and a rounded shrunken morphology. In contrast, cells overexpressing Myc-tagged CK2α showed a slightly but consistently higher value of [3H]thymidine incorporation than the controls. The defect in cell growth and changes in morphology caused by Myc-CK2β overexpression were partially rescued by coexpression of Myc-tagged CK2α. In parallel to the studies in CHO cells, the stable transfection of Myc-CK2α and Myc-CK2β subunits was achieved in 3T3 L1 fibroblast cells. Similarly, the ectopic expression of Myc-CK2β, but not Myc-CK2α, caused a growth defect. By measuring [3H]thymidine incorporation, it was found that expression of Myc-CK2β prolonged the G1 phase and inhibited up-regulation of cyclin D1 expression during G1. In addition, a lower mitotic index and lower mitotic cyclin-dependent kinase activities were detected in Myc-CK2β-expressing cells. Detailed analysis of stable cells that were synchronously released into the cell cycle revealed that the expression of Myc-CK2β inhibited cells entering into mitosis and prevented the activation of mitotic cyclin-dependent kinases. Taken together, results from both transient and stable expression of CK2 subunits strongly suggest that CK2 may be involved in the control of cell growth and progression of the cell cycle.

Casein kinase 2 (CK2) is a ubiquitous, multifunctional eukaryotic serine/threonine protein kinase that phosphorylates many different substrates including metabolic enzymes, structural proteins, transcription factors, and proto-oncoproteins (1). The holoenzyme form of CK2 is a heterotetramer, composed of α, α’, and β subunits combined to form αββ, ααβ, and α’ββ. The α and α’ subunits are catalytically active, whereas the β subunit is thought to be a regulatory subunit that stimulates the catalytic activity of α or α’ subunits and may also influence substrate specificity (for reviews, see Refs. 1–4). CK2 exhibits remarkable evolutionary conservation of primary structure in all eukaryotes from yeast to human, e.g. the identity of amino acid sequences of α and β subunits between human and Drosophila melanogaster is 90 and 88%, respectively. The amino acid sequences of the β subunits of human, pig, and chicken are even identical, underscoring this point (5–6).

The physiological role of CK2 has been explored in yeast and in a number of mammalian cell types, and these studies suggest that the enzyme is involved in cell growth and progression of the cell cycle. For example, genetic studies in Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Dictyostelium discoideum (7–9) showed that CK2 activity is essential for cell viability, e.g. the simultaneous disruption of the genes encoding the catalytic subunits, cka1 and cka2, in S. cerevisiae is lethal (7). An essential role of CK2 in control of cell cycle progression has also been demonstrated in the yeast S. cerevisiae (10). Through the use of mutant strains temperature-sensitive for the CK2 gene, the function of CK2 during the cell cycle was analyzed. It was shown that following a shift to the nonpermissive temperature, the mutant strains arrested within a single cell cycle and showed a dual arrest phenotype consisting of 50% of cells in G1 and 50% cells in G2/M. Further analysis by flow cytometry of pheromone-synchronized cells confirmed that CK2 is required at a point beyond Start in G1 prior to S phase. Analysis of hydroxyurea-synchronized cells also confirmed that CK2 is needed for cells cycle progression in the G2/M phases in yeast (10).

Insofar as the role of CK2 in growth-related functions in mammalian cells is concerned, it has been shown that microinjection of antibodies directed against the β subunit inhibits cell cycle progression in response to serum stimulation in human IMR-90 cells (11). CK2 antisense treatment was found to inhibit cell growth stimulation (12) and block neuritogenesis in neuroblastoma cells (13). In experiments with a transgenic CK2α mouse model, the expression of CK2α, even when seen only at the mRNA level, caused a high predisposition for lymphoma formation, and coexpression with c-Myc resulted in the rapid development of leukemia (14).

The importance of CK2 on cell growth and cell cycle progression is also suggested by structural analysis of the enzyme and by the fact that a number of CK2 substrates are growth- and cell cycle-related (4, 21). The catalytic subunits of CK2, α, and α’, which are highly homologous, are closely related to the p34cdc2 family, whose activities are required for G1/S and G2/M.
transitions in the cell cycle (15). In addition, both types of subunits of CK2, i.e. the α and β subunits, can be phosphorylated by p34(Cdc2) in vitro and in intact cells during mitosis (16–18). Furthermore, p34(Cdc2) itself can be phosphorylated by CK2 (19). Cyclin B1, which binds to and activates p34(Cdc2) during mitosis, appears to be phosphorylated by CK2 at the sites that regulate its translocation during mitosis (20).

Despite the numerous findings that suggest a role for CK2 in the control of cell growth, direct evidence obtained by overexpression of this enzyme in cells is still lacking; it has only been previously overexpressed in COS cells, a cell line that normally would not show any phenotype. In the present study, the transient overexpression of the epitope-tagged CK2 subunits, Myc-CK2α and Myc-CK2β, in CHO cells and development of stable cell lines in 3T3 L1 fibroblasts has been achieved. To our knowledge, this is the first paper reporting the successful exogenous expression of CK2 in mammalian cell lines other than COS cells. In both cell systems, it was found that the expression of Myc-CK2β caused severe impairment of growth. An analysis of the 3T3 L1/Myc-CK2β stable cell lines showed that similar to what was observed in yeast (10), the growth inhibition appears to be linked to defects in the progression of the cell cycle.

Experimental Procedures

Plasmids

The Myc-tagged human CK2α and CK2β cDNAs were subcloned from pCK2α and pSC/CK2β plasmids (22, 23) into pcDNA3 from the BamHI and XhoI sites (for CK2α) and from BamHI and XhoI sites (for CK2β), respectively.

Antibodies

Polyclonal anti-CK2α A-14, monoclonal anti-cyclin B1 antibody GNS1, rabbit polyclonal anti-cyclin A antibody C-19, and rabbit polyclonal anti-cyclin B1 antibody H250 were obtained from Santa Cruz Biotechnology. Monoclonal anti-Myt antibody 9E10 was a gift from Dr. J. A. Cooper (University of Washington, Seattle). Polyclonal anti-CK2α and -β antisera were raised against synthetic peptides and were employed in our study as described elsewhere (24). Monoclonal anti-cdc2 antibody was from Transduction Laboratories. Monoclonal MPM2 antibody, which was raised against phosphoproteins during mitosis, was from Upstate Biotechnology.

Cell Culture, Transfection, and Preparation of Cell Lysates

Chinese hamster ovary cells (CHO cells) were cultured in 150-mm plates containing F10 medium with 10% fetal calf serum (FCS) and grown to confluency. A day prior to the transfection, the cells were trypsinized and plated after a one to two dilution and were allowed to grow for another day. For transfection, the cells were trypsinized, washed by centrifugation with growth medium followed by phosphate-buffered saline (PBS), resuspended in 0.5 ml of PBS, pH 7.4 (Life Technologies, Inc.), and transfected by electroporation (Gene Pulser, Bio-Rad) at 0.35 kV and 926 microfarads. After electroporation, 1 ml of growth medium was added quickly, and the cells were plated. Twelve hours later, the medium was changed, and cells were grown for another 24–36 h before being harvested by sonication in Buffer A (50 mM β-glycerophosphate, pH 7.3, 20 μM vanadate, 1 mM diithiothreitol (DTT), 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Transfection of CHO cells by using Lipofectin Reagent was also performed following a protocol from Life Technologies, Inc.).

Cell Counting

Briefly, cells were seeded at a density of 2500 cells/35-mm plate, with duplicate plates for each cell line. Every 24 h, the cells were trypsinized and counted using a hemocytometer (25).

3H/Thymidine Incorporation

For CHO cells, after electroporation, cells were seeded in 6-well/35-mm plates at a density of one-tenth of the total transfected cells per well. At 12 h after transfection, the transfection medium was removed, and the cells were washed three times with PBS and cultured in fresh medium (2 ml containing 1 μCi/ml [3H]thymidine 5′-triphosphate (NEN Life Science Products) for another 24 h. To eliminate the possibility that the cell density between plates might be different after transfection, which could cause variations in the value of 3H incorporation, two extra plates were plated and used to count the cell number for each transfection at the time when [3H]thymidine was added. For harvesting cells, the growth medium containing [3H]thymidine was removed, and the cells were then washed twice with PBS. The cells were rinsed twice with 2 ml of ice-cold 5% trichloroacetic acid and lysed by incubation in 1.5 ml of 0.25 M NaOH for 15 min at room temperature. 0.6 ml of lysates was used for counting [3H]thymidine incorporation.

Stable cell lines of 3T3 L1/Myc-α and 3T3 L1/Myc-β were plated growing 35-mm/6-well plates at a density of 5 × 104 cells/plate. After 24 h, the cells were starved for 36 h in 2 ml of DMEM containing 0.1% FCS, followed by growing in 10% FCS medium containing 1 μCi/ml [3H]thymidine for 18 h. The cells were harvested at various time points and 3H incorporation was measured as described for CHO cells. For all of the experiments, duplicate plates were used and mean values were taken.

Kinase Assays

Histone H1 Kinase Assay—The cyclin B1-associated p34(Cdc2) and the cyclin A-associated CDKs were co-immunoprecipitated from stable cell lines of 3T3 L1/Myc-α and 3T3 L1/Myc-β using the anti-cyclin B1 antibody, GNS1, and the anti-cyclin A antibody C-19, respectively. The CDK activities were measured using histone H1 (Sigma) as the substrate. Briefly, for asynchronous cells, the actively growing cells (60–70% confluent) were harvested and lysed by sonication in Buffer A. Then 500 μl of the crude cell lysates (1 mg of protein/ml of lysate) was incubated with either 1 μg of the anti-cyclin B1 antibody or the anti-cyclin A antibody for 3 h at 4°C. After this, 40 μl (50% slurry) of protein G-Sepharose (for cyclin B1 immunoprecipitates) or protein A-Sepharose (for cyclin A immunoprecipitates) was added and incubated for another 90 min. The immunoprecipitates were washed once with lysis buffer and three times with a wash buffer containing 50 mM Tris-Cl, pH 7.5, 0.25 mM NaCl, 10 mM MnCl2, and 1 mM DTT. Reactions were initiated by addition of 30 μl of an assay buffer containing 4 μg of histone H1, 20 μM MgCl2, 7 mM MnCl2, 150 mM NaCl, and 0.1 mM [γ-32P]ATP (2000 cpm/pmol ATP). The reactions were carried out for 30 min in an incubator shaker at 37°C and then stopped by addition of Laemmli sample buffer.2 Phosphorylation of histone H1 was analyzed by SDS-PAGE and autoradiography.

2 J. H. Wright, E. S. Munar, D. R. Jameson, P. Andreassan, R. Margolis, R. Seger, and E. G. Krebs, submitted for publication.

Expression of CK2 in Mammalian Cells

Monoclonal anti-Myt antibody 9E10 (10 μg) was added to 400 μl of crude cell lysates containing approximately 1 mg/ml total protein. The mixture was incubated for 2 h at 4°C. Then 40 μl of a mixture of protein A-Sepharose (Sigma) and protein G-Sepharose (Amersham Pharmacia Biotech) (1:1, 50% slurry) was added, and incubation was continued for another 90 min. The beads were spun down and washed 4 times by centrifugation in a wash buffer containing 50 mM Tris-Cl, pH 7.5, 0.15 M NaCl, and 2 mM EDTA. The immunoprecipitated Myc-CK2 proteins were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane and detected by immunoblotting with 9E10 hybridoma supernatant.

Cell Counting

The rate of stable clones of CK2 transfected 3T3 cells proliferation was measured by counting the number of cells after cells were plated. Briefly, cells were seeded at a density of 2500 cells/35-mm plate, with duplicate plates for each cell line. Every 24 h, the cells were trypsinized and counted using a hemocytometer (25).

Expression of CK2 in Mammalian Cells

Monoclonal anti-Myt antibody 9E10 (10 μg) was added to 400 μl of crude cell lysates containing approximately 1 mg/ml total protein. The mixture was incubated for 2 h at 4°C. Then 40 μl of a mixture of protein A-Sepharose (Sigma) and protein G-Sepharose (Amersham Pharmacia Biotech) (1:1, 50% slurry) was added, and incubation was continued for another 90 min. The beads were spun down and washed 4 times by centrifugation in a wash buffer containing 50 mM Tris-Cl, pH 7.5, 0.15 M NaCl, and 2 mM EDTA. The immunoprecipitated Myc-CK2 proteins were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane and detected by immunoblotting with 9E10 hybridoma supernatant.
autodigestion. Cells that were starved for 24 h by growth in a medium containing 0.1% FCS were used as a negative control.

**CK2 Assay**—The CK2 assays were performed using crude cell lysates (harvested by sonication in Buffer H) and a peptide substrate, RRRDDDDDDDA, as described previously (26, 27).

**Immunofluorescence Microscopy**

CHO cells that were transfected with the Myc-tagged CK2 constructs were seeded onto a 60-mm plate. At 24 h after transfection the cells were washed with PBS and fixed with –20 °C methanol for 5 min. The fixed cells were blocked with a TBST buffer (50 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 0.05% Tween) containing 5% goat serum for 1 h and then incubated overnight in blocking buffer containing a monoclonal anti-Myc antibody 9E10 (final concentration = 4.5 μg/ml). The plates were then washed six times with TBST and incubated with fluorescein isothiocyanate-conjugated secondary anti-mouse antibody (BioSource International, 1:1000 dilution) for 2 h. After washing five times with TBST, the plates were viewed using a Nikon Diaphot-TMD Inverted fluorescent microscope (28). The percentage of cells in elongated or rounded shape was determined for both the Myc-positive and Myc-negative (untransfected) populations. For determination of mitotic cells in 3T3 L1 stable clones, the same fixing and staining procedures were applied by using a monoclonal MPM2 antibody as the primary antibody. The percentage of mitotic cells was determined by counting MPM2-positive cells under the microscope.

**RESULTS**

**Transient Overexpression of Myc-CK2β Subunit in CHO Cells Inhibits Cell Proliferation and Results in an Abnormal Cell Morphology**—CHO cells were transiently transfected either with plasmids of each of the epitope-tagged CK2 subunits alone, pcDNA3/Myc-CK2α and pcDNA3/Myc-CK2β, or cotransfected with both subunits. The vector plasmid pcDNA3 was used as a control. In this system, by electroporation under the condition specified, the transfection efficiency was found to be greater than 50% as determined using the green fluorescence protein plasmid or by anti-Myc antibody immunostaining (data not shown). At 36–48 h after transfection, cells were harvested, and the overexpression of CK2 was detected by CK2 kinase assays (26, 27) and by immunoblotting using anti-CK2α and anti-CK2β antisera raised against the C-terminal peptides of each subunit of human CK2 (23). As illustrated in Fig. 1A, the Myc-tagged CK2 subunits migrated slower than the endogenous CK2 proteins on SDS-PAGE due to the Myc epitope. The approximate size for Myc-CK2α and Myc-CK2β is 60 and 44 kDa, respectively. Each Myc-CK2 subunit was strongly overexpressed in this system, with a more than 5-fold increase over the endogenous level of non-tagged protein. Cotransfection of Myc-CK2α with Myc-CK2β also gave good expression of each subunit (Fig. 1A). To examine if the overexpressed Myc-CK2 proteins were enzymatically active, the CK2 activities in lysates of cells that were transfected with Myc-CK2 subunits or cotransfected with combinations of them were determined using a peptide substrate, RRRDDDDDDDA. An appreciably higher CK2 activity was detected in cells that were transfected with Myc-CK2α than in cells that were transfected with pcDNA3 vector alone, showing that the overexpressed recombinant CK2α was active (Fig. 1B). A further activation of CK2 was detected when cells were cotransfected with Myc-CK2α and Myc-CK2β, implying that the Myc-tagged subunits are capable of combining to give holoenzyme forms that exhibit higher activities than that obtained with the free tagged CK2α subunits (Fig. 1B).

One distinctive phenotypic change observed for cells that were transfected with the Myc-CK2β construct was that they had a slower proliferation rate than the non-transfected controls. It took an additional 24 h for Myc-CK2β-expressing cells to reach confluence as compared with vector controls or Myc-CK2α-expressing cells. To examine the proliferation rate quantitatively, the relative levels of DNA synthesis were monitored by measuring [3H]thymidine incorporation. As anticipated, there were reproducible differences between cells that were transfected with Myc-CK2β and vector controls; the cells that were transfected with Myc-CK2β clearly showed values of [3H]thymidine incorporation that were approximately 50% of the vector control (Fig. 1C). Since the transfection efficiency was approximately 50%, it is likely that the Myc-CK2β expressing cells were not incorporating thymidine at all. Transfection of cells with Myc-CK2α resulted in slightly higher values of [3H]thymidine incorporation (approximately 20% higher) than the cells transfected with pcDNA3 vector alone. Transfection of these cells with Myc-CK2β again depressed [3H]thymidine incorporation but not to the levels reached with Myc-CK2β transfection without cotransfection of the α subunit (Fig. 1C).

The slow growing cells transfected with Myc-CK2β also showed changes in morphology. In the normal growing state, CHO cells exhibit a flat, elongated morphology, which allows them to become attached to the plate and proliferate. After transfection with the vector or with the Myc-CK2α, the cells fully recovered from the shock caused by electroporation within 24 h and resumed their normal morphology. However, with the expression of exogenous Myc-CK2β, a large population of cells had a rounded appearance, consistent with the morphology of growth-arrested cells. To further extend these observations, an immunostaining technique was applied using anti-Myc antibody with cells that had been transfected with the various Myc-tagged CK2 constructs. The percentages of cells in rounded or elongated shape were determined microscopically. In this case, most of the cells that overexpressed Myc-CK2β showed the round shape, whereas only a small fraction of the cells that overexpressed Myc-CK2α exhibited this morphology (Fig. 2 A and B). Cotransfection of CK2α with CK2β partially rescues this phenotype. To exclude further the possibility that the observed phenotype could be introduced by this specific transfection technique (electroporation), a different transfection method, lipofection, was applied. Similar results were obtained (data not shown). Of interest was the fact that in many ways the rounded cells overexpressing CK2β exhibited morphologic changes that occur in apoptosis. They had a shrunken appearance and eventually died. At 48 h after transfection, by co-staining with anti-Myc antibody and DNA staining with Hoechst dye, most of Myc-CK2β expressing cells were observed to exhibit chromosomal condensation and fragmentation, which is characteristic of apoptotic cell death (data not shown). On the other hand, very few cells that overexpressed Myc-CK2α showed such changes in the cell nuclei.

**The Stable Ectopic Expression of Myc-CK2β Subunit in 3T3 L1 Cells Inhibits Cell Proliferation**—In order to study long term cellular effects of expressing CK2 subunits, the stable expression of Myc-CK2β and Myc-CK2α was carried out using 3T3 L1 fibroblasts. After transfection of cells with the Myc-tagged CK2 subunits, multiple clones were examined for expression of proteins. Two approaches were applied as follows: one was by immunoblotting of crude cell lysates with anti-Myc antibody or anti-CK2 antibodies and another was by immunoprecipitation with anti-Myc antibody followed by immunoblotting with either anti-Myc antibody or with CK2 subunit antibodies. With the first method it was questionable whether or not the Myc-tagged CK2 subunits could even be seen regardless of which type of immunoblotting was employed, although the endogenous subunits were readily apparent, i.e. the blotting with anti-CK2 subunit antibodies (Fig. 3A). However, when initial immunoprecipitations were carried out, the expression of Myc-tagged CK2α and CK2β subunits was detectable (Fig. 3B). Multiple clones were examined, and 10 clones, 3 express-
ing Myc-CK2α at different levels and 7 expressing Myc-CK2β, again at different levels, were selected for further study (Fig. 3B and Table I). For the most part, these studies made use of clones a12, a13, b3, and b6, with clones b3 and a12 representative of the highest and clones a13 and b6 representative of a lower level of expression (Fig. 3B and Table I). Cells that were stably transfected with pcDNA3 vector were used as a control. The enzymatic activity of CK2 in these cells was also examined using a specific CK2 peptide substrate, RRRDDDSDDD. No significant change of CK2 activity was detected in any of these cell lines, consistent with the concept that the total concentration of CK2α-tagged plus untagged underwent very little change as a result of the expression of the Myc-tagged subunits.

The proliferation rates of the Myc-CK2α- and Myc-CK2β-expressing cell lines and pcDNA3 vector control cells were examined under normal growth conditions (10% FCS) by counting cell numbers. For this, the cells were seeded at a very low density and allowed to grow for 8 days. Every 24 h cells were trypsinized and counted. All of the CK2β clones examined showed a slower growth rate than the vector control cells. This is illustrated in Fig. 4 for clone b3, which had the highest expression level of Myc-β, and for clone b6, which had the lowest expression level. In contrast, Myc-CK2α transfectants showed a normal growth rate as compared with the vector control (Fig. 4).

Consistent with the observed growth inhibition, the β-ex-
Expression of CK2 in Mammalian Cells

**Fig. 3.** Stable expression of Myc-tagged CK2 subunits in 3T3 L1 fibroblasts. A, immunoprecipitation of Myc-tagged CK2 subunits in transduced 3T3 L1 cells. Crude lysates of 3T3 L1 cells expressing Myc-CK2α and Myc-CK2β and pcDNA3-transfected clone v were immunoprecipitated with polyclonal anti-CK2α and anti-CK2β antibodies. Endogenous CK2α and CK2β are indicated by arrows, as are the positions of Myc-CK2α and Myc-CK2β. An unidentified (“nonspecific”) band is also shown. The particular Myc-CK2α and β clones examined are defined in Table I. B, detection of Myc-tagged CK2 subunits in 3T3 L1 fibroblasts by immunoprecipitation. Myc-CK2α and Myc-CK2β were immunoprecipitated using a monoclonal anti-Myc antibody 9E10 and subjected to SDS-PAGE and immunoblotting by use of the same antibody. The pcDNA3-transfected clone v was used as control in each case.

pressing cells tended to lose the expression of the exogenous gene with passage number. For example, after 15 passages, the expression of Myc-CK2β could not be detected even in the β3 clone. These cells fully reverted to the normal growth phenotype (data not shown). In contrast, no obvious decrease in the expression of recombinant CK2α was detected. Therefore, all the data presented here were obtained from cells of early passages (less than 10 passages).

The Expression of Myc-CK2β in 3T3 L1 Cells Prolongs the G1 Phase Cell Cycle Progression and Negatively Regulates Cyclin D1 Expression—Since a slower growth rate was observed for the CK2β-expressing cells, flow cytometric analysis (FACS) was employed to determine whether there was any dysregulation in cell cycle progression. As illustrated in Table II, for actively growing asynchronized cells, both parental 3T3 L1 cells and the pcDNA3 vector control cells exhibited similar

FACS profiles, with approximately 30% of cells in G1 phase and 30% cells in G2/M phase. However, the β clones showed very different profiles. For clones examined, accumulation of G2/M peak was seen with the increasing expression level of Myc-CK2β. For β3 and β6, while β6 cells behaved more or less like the control cells, a much higher percentage of β3 cells was in the G2/M peak. This strong G2/M peak was not changed even after the cells were starved for 48 h (data not shown). This makes it very difficult to analyze further the effect of CK2β expression on cell cycle progression by FACS. Therefore, we had to perform other experiments, including [3H]thymidine incorporation and CDK assay, etc., to do cell cycle analysis.

Consistent with what was observed in the cell counting experiment (Fig. 4), [3H]thymidine incorporation assay for stable clones v, α12, α13, β3, and β6 also demonstrated a slower proliferation rate for Myc-CK2β cells. The values of [3H]thymidine incorporation by β3 and β6 were much lower than that of the vector cells or the two Myc-CK2α cells (Fig. 5A). Since the cells started in G0, and the serum-stimulated [3H]thymidine incorporation occurs when cells are in S phase of cell cycle, the
decreased thymidine incorporation in the β-clones suggested a possible G1 arrest. An analysis of the kinetics of cell cycle progression from G₀ to G₁ and then to S phase was carried out by measuring the time course of [³H]thymidine incorporation. The results for clones v and β₁ were shown in Fig. 5B. In this experiment, cells were starved for 48 h and then stimulated with 10% FCS in the presence of [³H]thymidine and harvested at different time intervals thereafter. For the vector control cells, there was a significant increase of [³H]thymidine incorporation starting at approximately 16 h after serum stimulation, indicating that cells were starting to enter S phase. This entry into S phase was confirmed by the appearance of peaks with a DNA content greater than 2 n by flow cytometric analysis (data not shown). Expression of Myc-CK2β delayed entry into S phase by approximately 2 h (Fig. 5B). Similar results were observed for other Myc-CK2β clones (data not shown).

One of the key regulators for G₁ progression in mammalian cells is cyclin D1, which associates with and activates CDK4/CDK6 activity in late G₁ phase in proliferating cells. We examined whether the level of cyclin D1 in actively growing asynchronous cells was affected by the expression of Myc-CK2 subunits. As shown in Fig. 6A, there was a clear reduction of cyclin D1 expression in both β₁ and β₆ as well as other Myc-CK2β-expressing clones (data not shown) as compared with the vector control and the Myc-CK2α clones, α₁₂ and α₁₃ (Fig. 6A). The effect of expressing Myc-CK2β on cyclin D1 levels was also seen when synchronized cells were used in the study. In these experiments cells were subjected to serum starvation in order to arrest them in G₀ and then stimulated with serum to enter the G₁ phase. The level of cyclin D1 at different time intervals after serum stimulation was examined (Fig. 6B). In vector control cells, the expression of cyclin D1 was very low in quiescent cells but increased appreciably after stimulation (4-fold by 14 h). However, expression of Myc-CK2β inhibited the serum-stimulated up-regulation of cyclin D1, with only a small increase of cyclin D1 expression after 14 h (1.1-fold). In contrast to what was seen with Myc-CK2β, the expression of Myc-CK2α did not suppress the up-regulation of cyclin D1 expression (data not shown).

The Expression of Myc-CK2β in 3T3 L1 Fibroblasts Reduces Mitotic Index and Mitotic CDK Activities in Asynchronous Cells—To help clarify the cell cycle changes that occurred in Myc-CK2 subunit-expressing cells, the percentage of cells in mitosis (mitotic index) was determined by immunostaining with a mitotic-specific monoclonal antibody, MPM2. The results are shown in Fig. 7A. Compared with vector control cells, the Myc-β-expressing cell lines had a significantly lower number of mitotic cells, whereas Myc-α-expressing cells had a slightly higher number of mitotic cells. The percentage of the mitotic cells counted for the vector control cells was approximately double that for β₁ and also significantly higher than for β₆; a lower number of mitotic cells were also seen with the other β clones, suggesting that the CKβ-expressing cells might have difficulty in entering mitosis.

As is widely recognized, the activation of the p34cdc2-cyclin B1 complex is a hallmark of mitosis. Together with the p34cdc2-cyclin B1 complex, activation of the p34cdc2-cyclin A complex also promotes cell entry into mitosis. In order to understand further the mechanism of the possible mitotic defect caused by the ectopic expression of CK2β, the mitotic CDK activities associated with cyclin B1 and cyclin A in asynchronous cells were measured. The cyclin B1 and cyclin A proteins were each immunoprecipitated from the cell lysates, and the activities of the associated CDKs were assayed using histone H1 as a substrate. Clones β₁ and β₆ showed reduced cyclin B1-associated Cdc2 and cyclin A-associated CDK activities as compared with the vector control and the two CK2α clones (Fig. 7B). In contrast to the CK2β cell lines, the CK2α cell lines exhibited similar or perhaps slightly higher mitotic CDK activities.

The Expression of the Myc-CK2β in 3T3 L1 Cells Reduces the Percentage of Mitotic Cells and Mitotic CDK Activities in Synchronized Cells—Since a G₁ arrest was suggested in the CK2β cells, a G₁/M phase cell synchronization procedure was performed to determine whether the lower mitotic index and the reduced mitotic CDK activities observed for asynchronous Myc-CK2β-expressing cells might also be contributed to by a G₂ arrest. For this study, cells were blocked at the G₁/S boundary using the DNA synthesis inhibitor, aphidicolin. After the removal of aphidicolin, cells enter into S phase synchronously and progress through G₂ and M phases. Cells were harvested

**TABLE II**

Percentage distribution of the 3T3 L1/CK2β cells in different phases of cell cycle determined by FACS analysis

| Clones    | % Fluorescence at cell cycle phase |
|-----------|-----------------------------------|
|           | G₁      | S       | G₂       |
| 3T3 L1    | 33.2    | 14.4    | 33.2     |
| pcDNA3 (V)| 36.2    | 13.4    | 27.2     |
| β₁        | 22.8    | 9.5     | 44.7     |
| β₂        | 42.9    | 12.0    | 30.0     |
| β₃        | 33.4    | 11.5    | 30.0     |
| β₆        | 22.9    | 8.5     | 42.0     |

**FIG. 5. Expression of Myc-CK2β inhibits DNA synthesis.** A. thymidine incorporation into DNA in serum-stimulated 3T3 L1 cells. B. kinetics of [³H]thymidine incorporation into DNA was measured. The value of [³H]thymidine incorporation of the vector control, v, was taken as 100%, and the others were calculated as percent [³H] incorporation. Mean values ± S.D. from at least three experiments is reported. B. kinetics of [³H]thymidine incorporation for the vector control, v, and the stable clone β₆. Cells were plated, starved for 48 h, and stimulated with 10% FCS medium containing [³H]thymidine. At the indicated hours after serum stimulation, cells were harvested, and [³H]thymidine incorporation was determined.
at different times, and the percentage of mitotic cells was determined by counting cells that stained positively with MPM2 antibody. As illustrated in Fig. 8A, the percentage of MPM2-positive cells was the highest 8 h after the removal of aphidicolin for both vector controls (v) and the β3 clone. The clone β3 exhibited a much lower percentage of mitotic cells throughout the time course.

Detailed analysis of the cyclin B1-associated p34cdc2 activities and cyclin A-associated CDK activities for stable clones v and β3 were carried out for the synchronized cells (Fig. 8, B and C). Consistent with the MPM-2 cell-staining data, the expression of Myc-CKβ resulted in lower p34cdc2/cyclin B1 activity (Fig. 8B) and inhibited the activation of CDK/cyclin A activity (Fig. 8C). Almost no activation of CDK/cyclin A was observed for β3 after aphidicolin was removed and the cells entered mitosis (Fig. 8C).

**DISCUSSION**

The role of CK2 in the control of cell growth and cell cycle progression has been suggested by a number of studies in yeast (reviewed in Ref. 4), but direct evidence for such a role in mammalian cells is lacking due to the difficulty of expressing this enzyme in cells. In this paper, for the first time, we report the successful expression of epitope-tagged CK2 subunits in two cell lines by transient (in CHO cells) and stable expression (in 3T3 L1 cells) methods, and we have studied the effects of the individual subunits on proliferation and cell cycle progression. Results using both systems support each other; expression of Myc-CKβ caused growth inhibition and abnormal cell morphology, but expression of Myc-CK2α resulted in slower growth and had no significant effect on cell growth in 3T3 L1 cells. The growth inhibition caused by Myc-CKβ expression was not due to the Myc epitope, since the same phenotype was also observed in both cell lines that were transfected with an untagged pcDNA3/CK2β plasmid and, as noted above, was not seen in cells that expressed Myc-CK2α. This finding was similar to the work reported earlier in *S. pombe*.

![Image](https://example.com/image.png)

**Fig. 6. Expression of the Myc-CKβ subunit affects the expression level of cyclin D1.** A, Myc-CKβ cells have reduced level of cyclin D1 expression. Actively growing cells were harvested, and cell lysates were prepared. Expression of cyclin D1 was determined using a polyclonal rabbit anti-cyclin D1 antibody H295 (Santa Cruz Biotechnology). Endogenous CK2α expression was determined using a polyclonal anti-CK2α antibody to ensure equal loading. B, expression of CK2β inhibited up-regulation of cyclin D1 expression during G1 phase in synchronized β3 cells. Cells were synchronized to G0 by starvation and then stimulated with 10% FCS medium. After 0, 4, 6, 8, 10, 12, and 14 h, cells were harvested and examined for the expression of cyclin D1.

![Image](https://example.com/image.png)

**Fig. 7. Asynchronous Myc-CKβ clones exhibit reduced mitotic index and mitotic CDK activities.** A, percent mitotic cells for asynchronous stable Myc-CK2α and Myc-CK2β clones. Cells were washed with PBS, fixed with methanol, and stained with a mitotic-specific monoclonal antibody (anti-MPM2). The percentage of mitotic cells was determined by microscopically counting MPM2-positive cells in several random microscopic fields. Average data were taken from at least three plates for each clone, and standard errors were calculated. B, change of the mitotic CDK activities in asynchronous Myc-CK2β-expressing cells. Cyclin B1 and cyclin A were immunoprecipitated from the lysates of actively growing cells: v, β3, β3, α12s, and α13s. The cyclin B1-associated p34cdc2 activity and the cyclin A-associated CDK activities were determined using histone H1 as the substrate by mixing 4 μg of histone H1 and 25 μl of [γ-32P]ATP with the immunoprecipitates and incubating at 37 °C for 30 min. Phosphorylation of histone H1 by CDKs was analyzed by SDS-PAGE.

(29), in which overexpression of ckb1, the *S. pombe* CK2β subunit, inhibited cell growth and cytokinesis. It was of considerable interest that the growth changes observed in the 3T3 L1 cells expressing Myc-CKβ occurred even though the expression levels were very low as compared with endogenous subunit concentrations. It should be noted, however, that changes in the concentration of the total amount of CK2α and CK2β (tagged and endogenous) would have to be more than 1–2% to be detectable.

By further analysis of the stably transfected CK2 cells, it was found that the slow proliferation caused by the expression of recombinant Myc-CKβ appears to be linked to defects in cell cycle progression. Examination of the DNA synthesis in CK2β clones revealed decreased values of [3H]thymidine incorporation when quiescent cells were stimulated with serum to re-enter into G1 phase and then progressed into S phase. Expression of the CK2β subunit delayed entry into S phase for at least 2 to 3 h. Moreover, loss of serum-induced cyclin D1 expression in CK2β clones was also correlated with G1 arrest. It is well established that accumulation of cyclin D1 in G1 in response to mitogen is required for progression through the restriction point and entry into S phase. Therefore, the growth inhibition caused by CK2β expression appeared to affect cells in the G1 phase at a time before the restriction point.

In addition to the G1 effect, a lower mitotic index and reduced mitotic CDK activities were found in asynchronous Myc-CKβ-expressing cells. This could also result from growth defect in G1 phase. However, by applying a G1/M phase cell synchronization procedure, in which cells were synchronously progressed through S phase then G2 and M phases, a lower mitotic index and reduced mitotic CDK activities in β3 and
Expression of CK2 in Mammalian Cells

other β clones were also seen. This may indicate that the expression of CK2β caused a defect in cell mitosis. It appears that the expression of Myc-CK2β has an effect on cell cycle progression at two points, G1 and G2/M, a similar result as had been obtained in yeast (10).

Several hypotheses can be proposed to explain the effect of growth inhibition and cell cycle changes observed in the Myc-CK2β-expressing cells. First, CK2β appears to be important for substrate specificity of the CK2α subunit (4). Many CK2 subunits interact with the holoenzyme through its β subunit, e.g. p53 (30), DNA topoisomerase II (31), and the nuclear protein Nopp140 (32). A number of proteins are CK2 targets during cell cycle progression, including several cytoskeletal proteins, whose phosphorylation by CK2 might contribute to the structural rearrangements underlying mitosis, and some transcription factors that might be important for the transcription of cell cycle-related proteins (33, 34). Disruption of CK2 activity in budding yeast S. cerevisiae resulted in accumulation of cells at both G1 and G2/M phases (10). Likewise, expression of Myc-CK2β also caused a similar phenotype as that which occurred in yeast when CK2 activity was disrupted. It is possible that Myc-CK2β may interfere with the activity of the holoenzyme by competing for binding to important CK2 substrates that are critical for cell cycle progression. However, in this model, a perplexing question is why such a very small amount of the exogenously expressed Myc-CK2β could neutralize the free α, causing a defect in cell growth. This mechanism is highly favored from our work based upon the observation that such a very small amount of the exogenously expressed CK2β protein functions efficiently in inhibiting cell growth in 3T3 L1 cells. Furthermore, it is also suggested from the observation that transient expression of CK2α increased the proliferation rate of CHO cells and that coexpression of CK2α and CK2β in CHO cells only partially rescued the growth inhibition of Myc-CK2β, although the level of CK2 holoenzyme was very high.

Since CK2 is always found as a tetramer under preparative conditions, the critical question still remains as whether or not the free subunits exist inside cells. In fact, the existence of free CK2β subunits has been demonstrated in several organisms, such as in Zea mays (40), D. discoideum (9), and possibly in mammalian cells (41). The growth-promoting function of the free CK2α form has also been documented. Drosophila CK2α, which does not bind to yeast CK2β, rescues the yeast mutant cell in which both CK2 catalytic subunits, cka1 and cka2, were disrupted (7). Transgenic mice overexpressing CK2α exhibit an increased chance of tumorigenesis (14). Although the holoenzyme CK2 displays a higher catalytic activity toward most commonly used substrates such as casein, the RRREDVDDD peptide, and others, there are a few examples that monomeric CK2α can be more active toward certain substrates, such as calmodulin. Tetrameric CK2 does not phosphorylate calmodulin under normal phosphorylation conditions, whereas monomeric CK2α phosphorylates it efficiently (42, 43). It is possible that there are other growth-related proteins that are better substrates of free CK2α subunit than for the holoenzyme. The identification of such substrates for CK2α subunit might provide useful in-

FIG. 8. Expression of the Myc-CK2β subunit affects cell mitosis in synchronized cells. Stable clones v and β were plated at low density, starved for 48 h, and synchronized to the G1/S boundary by culturing in medium containing 10% FCS and 1 µg/ml aphidicolin for 18 h. After extensive washing, the cells were cultured in fresh medium and were then either fixed or harvested at different time intervals. The fixed cells were stained with the MPM2 antibody, and the percentage of mitotic cells were determined by counting MPM2-positive cells microscopically (4). The cyclin B1-associated pS4/tyr2 activities (B) and cyclin A-associated CDK activities (C) at different time point were measured for immunoprecipitates of cyclin B1 or cyclin A using histone H1 as the substrate (see “Experimental Procedures”). Histone H1 phosphorylation by CDKs illustrated as percent volume from densitometer readings was analyzed by SDS-PAGE and autoradiography.
Expression of CK2 in Mammalian Cells

formation as to targets of the enzyme critical in its role in control of cell cycle progression.

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