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Inducible Expression of the Regulatory Protein Kinase CK2β Subunit: Incorporation Into Complexes With Catalytic CK2 Subunits and Re-Examination of the Effects of CK2β on Cell Proliferation

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Abstract The regulatory subunit of protein kinase CK2, designated CK2β, exists both free in cells and in complexes with the CK2 catalytic subunits. Growing evidence suggests that CK2β has functions dependent and independent of the CK2 catalytic subunits. There have been indications that CK2β has functions associated with DNA damage responses and in the control of cell proliferation. For example, transient and stable constitutive overexpression of CK2β in mammalian cells was previously shown to perturb cell cycle progression and to attenuate proliferation. To systematically investigate the molecular mechanisms responsible for these effects of CK2β on cell proliferation, we generated human osteosarcoma U2OS cell lines with tetracycline-regulated expression of CK2β. Increased expression of CK2β results in increases in total cellular CK2 activity, but no changes in cell cycle profiles or proliferation. Furthermore, following exposure to ultraviolet radiation, p53 induction was identical regardless of the levels of CK2β in cells. Mouse 3T3-L1 cells stably transfected with CK2β also showed no alterations in cell proliferation. The differences between these results and those previously reported emphasize the complex nature of CK2β and its cellular functions. Furthermore, these results indicate that increased expression of CK2β is not by itself sufficient to effect alterations in cell proliferation. J. Cell. Biochem. 84: 84–99, 2002.

Key words: CK2; regulatory; proliferation; inducible; osteosarcoma; fibroblast; tetracycline; cell cycle

Protein kinase CK2 (CK2) is an ubiquitous serine/threonine kinase that is important in many aspects of cellular regulation [Litchfield and Luscher, 1993; Allende and Allende, 1995; Pinna and Meggio, 1997; Glover, 1998; Guerra and Issinger, 1999]. Genetic studies in *Schizosaccharomyces pombe* [Roussou and Draetta, 1994], *Saccharomyces cerevisiae* [Chen-Wu et al., 1988; Padmanabha et al., 1990], and *Dictyostelium discoidium* [Kikkawa et al., 1992] indicate that CK2 is essential for viability. In tumor cells, CK2 expression levels have been observed to be dysregulated [Munstermann et al., 1990; Stalter et al., 1994; Yenice et al., 1994; Faust et al., 1996]. Studies using mouse BALB/c fibroblasts show that CK2 can collaborate with *Ha*-Ras to induce transformation [Orlandini et al., 1998]. Additionally, transgenic mouse models highlight the oncogenic potential of CK2 and demonstrate cooperativity between CK2 and c-Myc or with the loss of p53 [Seldin and Leder, 1995; Landesman-Bollag et al., 1998]. Collectively, these studies suggest that CK2 has important cellular functions as illustrated by the observation that perturbations in CK2 levels can have profound effects.

Abbreviations used: BCA, bicinchoninic acid; BSA, bovine serum albumin; CK2, casein kinase 2; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiothreitol; EDTA, ethylenediamine tetra-acetic acid; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; MCS, multiple cloning site; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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CK2 is generally comprised of two catalytic isozymes, designated CK2α and CK2α', and the regulatory subunit designated CK2β [Gietz et al., 1995; Graham and Litchfield, 2000]. The individual subunits of CK2 are highly conserved between different organisms highlighting their crucial importance in cellular function. In mammalian cells, CK2 exists as heterotetrameric (i.e., αα'ββ), or homotetrameric (i.e., ααββ or α'α'ββ) complexes. There is also mounting evidence suggesting that catalytic subunits can exist in the absence of CK2β [Heriche et al., 1997] and that CK2β exists in cells in the absence of CK2α or CK2α' [Luscher and Litchfield, 1994; Guerra et al., 1999]. The catalytic isozymes are responsible for serine/threonine phosphorylation of CK2 targets while the regulatory CK2β has a number of modulatory functions associated with the stability, activity, and specificity of CK2α and CK2α' in the holoenzyme complex [Guerra and Issinger, 1999]. Evidence also strongly suggests that CK2β has functions independent of the CK2 tetramer complex through its interaction with numerous cytoplasmic and nuclear cellular proteins such as CD5 [Raman et al., 1998], c-Mos [Chen and Cooper, 1997; Chen et al., 1997], Nopp140 [Li et al., 1997], A-Raf [Boldyreff and Issinger, 1997; Hagemann et al., 1997], p21 [Gotz et al., 1996], and p53 [Filhol et al., 1992; Appel et al., 1995; Prowald et al., 1997; Schuster et al., 1999].

The cellular functions of CK2β have been extensively studied through genetic studies in *S. cerevisiae* [Bidwai et al., 1994; Reed et al., 1994; Bidwai et al., 1995; de Nadal et al., 1999] and *S. pombe* [Roussou and Draetta, 1994]. In *S. cerevisiae* strains lacking either or both regulatory subunits, designated CKB1 and CKB2, the cells exhibited wild-type behavior except that they were sensitive to high concentrations of Na^+ and Li^+ cations. Strains overexpressing either CK2β subunit exhibited a normal phenotype. On the other hand, *S. pombe* strains engineered to overexpress CKB1 displayed a multiseptated phenotype with an inhibition of cell growth and cytokinesis while a disruption of the *ckb1* gene in *S. pombe* caused abnormalities in cell shape. Overall, these studies emphasize the complex nature of CK2β and its cellular functions.

Protein kinase CK2β has been implicated in many cellular events including DNA damage signaling pathways [Teitz et al., 1990; Toczyski et al., 1997], cell cycle progression [Chen and Cooper, 1997], and cellular proliferation [Lorenz et al., 1993; Li et al., 1999]. For example, introduction of a cDNA encoding the CK2β subunit into xeroderma pigmentosum cells could confer a partial UV resistance phenotype. In *S. cerevisiae*, the CK2β subunit along with CDC5 was identified in a screen for cells that possessed an adaptation defect at the G2/M phase transition due to a single double-stranded DNA break. There is also evidence at the molecular level that CK2β could participate in these events. For example, CK2β has been shown to interact with p53 and to inhibit its binding to p53-responsive promoters. This is an intriguing finding because p53 is important for checkpoint control at the G1/S and G2/M phase transitions of the cell cycle. However, the exact mechanism for how CK2β participates in these p53-mediated events remains poorly understood. CK2β also appears to modulate cell cycle progression in *Xenopus laevis* as increased expression of CK2β inhibited the serine/threonine kinase Mos and subsequent meiotic maturation [Chen and Cooper, 1997; Chen et al., 1997]. A role of CK2β in cellular proliferation has also been recently demonstrated in mouse fibroblast 3T3-L1 and Chinese hamster ovary cells. Transient and stable transfection of these cell lines with epitope-tagged CK2β resulted in a 1.5-fold increase in overall CK2 activity, an attenuation of cell proliferation and perturbations at specific stages of the cell cycle including mitosis and G1 [Li et al., 1999].

Based on the study of Li et al. [1999] demonstrating a profound effect of increased CK2β expression on cell proliferation, we set out to develop mammalian cells with regulated expression of CK2β to serve as a model for a systematic investigation of the mechanisms by which CK2β affects proliferation. To achieve this objective, we developed human U2OS osteosarcoma cells with the expression of *myc*-CK2β under the control of tetracycline. Total CK2β levels and CK2 activities were significantly increased in these cells following induction of *myc*-CK2β. Furthermore, our studies indicated that *myc*-CK2β is competent to enter complexes with catalytic CK2 subunits. However, despite the increases in CK2β levels and CK2 activity in these cells, we did not observe any dramatic phenotypic alterations nor did we observe alterations in proliferation or responses to UV. Transfection of mouse 3T3-L1 cells with

constructs encoding *myc*-CK2β also failed to bring about alterations in cell proliferation indicating that the effects that we observed were not unique to the U2OS cells. The differences between our results and those previously reported [Teitz et al., 1990; Li et al., 1999] further emphasize the complex nature of CK2β and its cellular functions. Furthermore, our results indicate that increased expression of CK2β is not by itself sufficient to effect alterations in cell proliferation or responses to UV.

MATERIALS AND METHODS

Plasmid Constructs

The *myc*-CK2β insert was digested from pRc/CMV-*myc*-CK2β [Penner et al., 1997] with *Not*I and *Apa*I and blunt-end ligated into the *Eco*RV site (MCS I) of the bi-directional pBI plasmid (Clontech) to generate pBI/*myc*-CK2β [Gossen and Bujard, 1992; Baron et al., 1995; Vilk et al., 1999]. A second *myc*-CK2β insert was digested from pRc/CMV-*myc*-CK2β with *Not*I and *Bsp120*I and ligated into the *Not*I site (MCS II) of pBI/*myc*-CK2β to generate pBI/*myc*-CK2β/*myc*-CK2β, designated pGV10. All constructs were verified by sequencing.

Antibodies

Polyclonal anti-CK2α antiserum directed against the C-terminal synthetic peptide, $\alpha^{376-391}$, and polyclonal anti-CK2β antiserum directed against the C-terminal synthetic peptide $\beta^{198-215}$ have been previously described [Litchfield et al., 1991,1992]. The hybridoma producing the 9E10 monoclonal antibodies directed against the *myc* epitope was injected into mice and ascites fluid was collected and fractionated by ammonium sulfate precipitation. Monoclonal anti-human p53 (DO1) was purchased from Santa Cruz Biotechnology. Goat anti-rabbit (GAR) and goat anti-mouse (GAM) secondary antibodies conjugated to horse-radish peroxidase (HRP) were purchased from Bio-Rad. Fluorescein Isothiocyanate (FITC)-conjugated GAM was purchased from Sigma.

Cell Culture and Transfections

UTA6 cells were derived from the human osteosarcoma cell line U2OS and express the tetracycline-regulated transcriptional activator fusion protein (kind gift from Dr. Christoph Englert, Forschungszentrum Karlsruhe, Germany). The UTA6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, antibiotic supplements (0.1 mg/ml streptomycin and 100 U/ml penicilin), and 460 μg/ml Geneticin G418 (Life Technologies Inc.). UTA6 cells were seeded into 6-well dishes and cotransfected with 1 μg pTK-hyg plasmid (Clontech) and 4 μg of pGV10 per well using the *Fugene 6* reagent (Roche Molecular Biochemicals) following the manufacturer's instructions. After 48 h, the cells were pooled and seeded into 150-mm culture dishes and grown in growth medium containing 1.5 μg/ml tetracycline (Sigma), 460 μg/ml G418, and 500 μg/ml hygromycin (Roche Molecular Biochemicals). Isolated colonies were picked using cloning cylinders after 10–14 days, grown in drug selection, and tested for inducible expression of *myc*-CK2β using Western blotting. Two cell lines designated GV10.11 and GV10.15 exhibiting regulated expression of *myc*-CK2β were selected for experimentation and maintained in a 37°C incubator with a 10% CO_2 atmosphere.

Mouse fibroblast 3T3-L1 cells that express the tetracycline-regulated transcriptional activator fusion protein were kindly provided by Dr. Bernard Lüscher (Institut für Molekularbiologie, Medizinische Hochschule, Hannover, Germany). The culture conditions for 3T3-L1 cells were identical to the UTA6 cells. To generate stably transfected cell lines, 3T3-L1 cells were seeded in 6-well dishes and cotransfected with 1 μg pTK-hyg plasmid with 4 μg of pGV10 plasmid per well using *ExGen 500* transfection reagent (MBI Fermentas) according to manufacturer's instructions. After 48 h, the cells were pooled and transferred into 10-cm dishes containing growth medium with 1.5 μg/ml tetracycline, 212 μg/ml G418, and 275 μg/ml hygromycin. Forty-five isolated drug-resistant colonies were picked as above and tested for expression of *myc*-CK2β using Western blotting. Three cell lines, RD1, RD3, and RD41, exhibiting detectable levels of *myc*-CK2β expression were chosen for further experimentation.

Cell Extracts and Immunoprecipitations

For kinase assays, GV10.15 cells were seeded in 10-cm dishes with or without 1.5 μg/ml tetracycline and allowed to grow for 48 h. The cells were then washed twice in PBS, once in kinase assay buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA) and then extracted

with 500 μl of kinase assay buffer supplemented with 1 mM DTT, and protease/phosphatase inhibitors (30 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF, and 1 mM Na_3VO_4). The cells were sonicated 2×10 s and were then centrifuged at 55,000 rpm in a Beckman TLA100.2 rotor for 20 min at 4°C. The supernatants were collected and either used fresh or kept at −80°C for later use.

For immunoprecipitations, GV10.15 cells were seeded as described above and expression of *myc*-CK2β induced for a period of 48 h. The cells were washed twice with PBS, once with immunoprecipitation buffer without Nonidet P-40 lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl), and lysed in immunoprecipitation buffer containing 1% Nonidet P-40 and all the inhibitors described above. The extracts were sonicated 2×10 s and were centrifuged using the same parameters as above. The supernatants were collected and protein concentrations were determined using the BCA assay (Pierce) with BSA as a standard. One milligram of protein was aliquoted into fresh tubes and normalized for volume. Twenty microliters of a 50% slurry of Protein-A Sepharose beads (Sigma) and either 0.5 μl of 9E10 antibody ascites or 1 μl of anti-CK2α antiserum were added to the protein supernatants. The immunoprecipitations were allowed to tumble for 1 h at 4°C. The beads were then collected by centrifugation and washed four times with 1% Nonidet P-40 lysis buffer and finally suspended in 50 μl Laemmli buffer with 10% β-mercaptoethanol. The 9E10 and CK2α immunoprecipitates were then analyzed on Western blots.

To monitor protein expression in total cellular lysates, the U2OS and 3T3-L1 cells were washed twice in PBS and lysed in 500 μl of T-buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2% Nonidet P-40, 0.5% Deoxycholate, 0.2% SDS, plus all the inhibitors as described above). The lysates were treated as described above except that centrifugation was at 95,000 rpm for a period of 25 min.

Kinase Assay

Seven micrograms of total protein from GV10.15 cells were assayed using the synthetic peptide substrate, RRRDDDSDDD, as described previously [Litchfield et al., 1990]. To monitor assay linearity, assays were performed for both 5 and 10 min at 30°C in a final reaction volume of 30 μl containing kinase assay buffer and 0.1 mM [γ-^{32}P]-ATP (specific activity 500–1,000 cpm/pmol) and 0.1 mM RRRDDDSDDD substrate. The reactions were initiated by the addition of lysate and terminated by spotting 20 μl of each reaction on P81 phosphocellulose paper as described previously. The papers were washed four times with 1% phosphoric acid, once with 95% ethanol and dried overnight. The P81 papers were then immersed in scintillant and counted in a Beckman LS 5801 scintillation counter. The kinase assays were performed in duplicate under linear conditions.

Ultraviolet Irradiation Treatment

GV10.15 cells were seeded into 150-mm plates and expression of *myc*-CK2β induced for 48 h. In preparation for UV treatment, media was removed and 1 ml of PBS was added to the plates. The cells were exposed to either 10 or 40 J/m^2 UVC radiation using a UV Stratalinker. After UV treatment, fresh media was added to the cells, and the cells were allowed to recover at 37°C in an atmosphere of 5% CO_2. At various times (i.e., 0, 8, 24, and 48 h post UVC irradiation), GV10.15 cells were collected as described above. Cells were extracted in T-buffer and analyzed on Western blots to examine p53 and *myc*-CK2β expression profiles.

Western Blotting

Equal amounts of protein lysates derived from U2OS and 3T3-L1 cell lines were subjected to SDS–PAGE [Laemmli, 1970] at a constant voltage of 200 V for 45 min. The proteins were then transferred to PVDF for 1 h at a constant voltage of 15 V in blotting buffer (25 mM Tris-Cl, 190 mM glycine, 20% methanol) [Towbin et al., 1979] using the Trans-blot Semi-Dry Electrophoretic transfer apparatus (Bio-Rad). The PVDF membranes were blocked in 1% BSA in 0.05% Tween-20 TBS (TBST) for 2 h at 4°C. The blots were then washed twice with TBST and incubated overnight at 4°C with either anti-CK2α (1:5,000), anti-CK2β (1:10,000), or anti-p53 (DO1) (1:1,000). Blots were then washed 3 times for 10 min with TBST and incubated with either HRP-GAM or HRP-GAR in TBST with 1% BSA for 1 h at room temperature. The PVDF membranes were subsequently washed 3 times for 10 min with TBST and once for 5 min with TBS prior to visualization using chemiluminescent substrate (Pierce). For 9E10 blots, all solutions were in 0.1% Tween-20/PBS (PBST)

instead of TBST and the 9E10 antibody concentration used was 1:2,000. Ponceau S staining demonstrated equivalent levels of blotted protein in each sample.

Immunofluorescence

GV10.15 cells (1×10^5 cells/well) were seeded in 6-well dishes on coverslips. On the next day, expression of *myc*-CK2β was induced by removal of tetracycline for a period of 48 h. The GV10.15 cells were then fixed onto the coverslips with 3.7% formaldehyde in PBS for 30 min at 37°C. The cells were then washed twice with PBS and subsequently permeablized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The Triton X-100 solution was washed out with 2×5 min PBS and 9E10 antibody (1:600) in PBS with 5% FBS was then added for 1 h at 37°C. The GV10.15 cells were washed 2×5 min with PBS and FITC-conjugated GAM (1:1,000) in PBS with 5% FBS was added for 1 h at room temperature in the dark. Four to five minute PBS washes were performed after the incubation of secondary antibody. Cells were then counter-stained with 0.2 μg/ml of DAPI (Sigma) for 10 min. The coverslips were mounted onto slides in mounting medium containing 100 mM Tris-Cl (pH 8.0), 90% glycerol, and 1 mg/ml phenylenediamine (Sigma) and viewed using a fluorescent microscope. Images were captured using a Leica DM IRBE fluorescent microscope controlled by OpenLab 2.2.5 Imaging software.

Cell Proliferation Assays and Cell Cycle Analysis

Osteosarcoma and 3T3-L1 cells (2×10^4 cells/well) were seeded in 6-well dishes in the presence of 1.5 μg/ml tetracycline. On the next day, expression of *myc*-CK2β was induced in GV10.15 and RD1, RD3, and RD41 cells by extensively washing with PBS. At the indicated times, the cells were harvested with PBS containing 5 mM EDTA and trypan blue was added at a 1:1 ratio. An aliquot was counted with a hemocytometer and the number of viable cells was recorded in triplicate.

For cell cycle analysis, GV10.15 cells (2×10^5 cells/dish) were seeded in 10-cm dishes in the presence of 1.5 μg/ml tetracycline. On the next day, the cells were extensively washed with PBS and the media lacking tetracycline was added to induce expression of *myc*-CK2β. At the indicated times, the cells were collected using PBS containing 5 mM EDTA, resuspended in PBS containing 2 mM EDTA and subsequently fixed by the drop-wise addition of 95% ethanol. Cells were incubated overnight at 4°C and on the next day they were resuspended in 600 μl of 50 μg/ml propidium iodide (Sigma), 0.1% sodium citrate, 0.1% Triton X-100, 0.1 mg/ml DNase-free RNaseA and incubated in the dark for 20 min. Thirty-five thousand cells were analyzed on a Becton Dickinson FACS analyzer at a flow rate of less than 200 cells/s. Cell cycle profiles were generated using CellQuest 3.1 analysis software. In parallel, lysates were collected at each time point to monitor *myc*-CK2β expression.

Protein Phosphatase Treatment

Total cell lysates (50 μg) from cells expressing *myc*-CK2β were incubated in Lambda phosphatase reaction buffer (50 mM Tris-Cl (pH 7.5), 0.1 mM Na_2EDTA, 5 mM DTT, 0.01 % Brij 35, and 2 mM $MnCl_2$) supplemented with or without 400 Units of Lambda phosphatase (Cell Signaling Technology) for 30 min at 30°C. Following this incubation, Laemmli sample buffer was added to stop the reaction and the samples were resolved on a 12% SDS–PAGE gel and immunoblotted with *myc* (9E10) antibodies. For these experiments, total cell lysates were prepared with lysis buffer lacking phosphatase inhibitors.

RESULTS

Inducible Expression of CK2β

The regulatory subunit CK2β subunit has been implicated in cell cycle progression, cell proliferation, and DNA damage. Consequently, we generated tetracycline-inducible cell lines in order to systematically examine the mechanisms by which this CK2 subunit influences these cellular events. The human osteosarcoma U2OS cell lines express *myc*-tagged CK2β under the control of the tetracycline trans-activator protein using a bi-directional plasmid that encodes two copies of *myc*-CK2β (Fig. 1A). Cell lines designated GV10.11 and GV10.15 were chosen for further investigation because they exhibited regulated *myc*-CK2β expression. After 24 h of induction, expression of CK2β in whole cell lysates was examined by Western blot using antibodies against the *myc* epitope tag (Fig. 1B) or antibodies directed against the C-terminus of CK2β (Fig. 1C). The presence of tetracycline in the medium completely inhibits

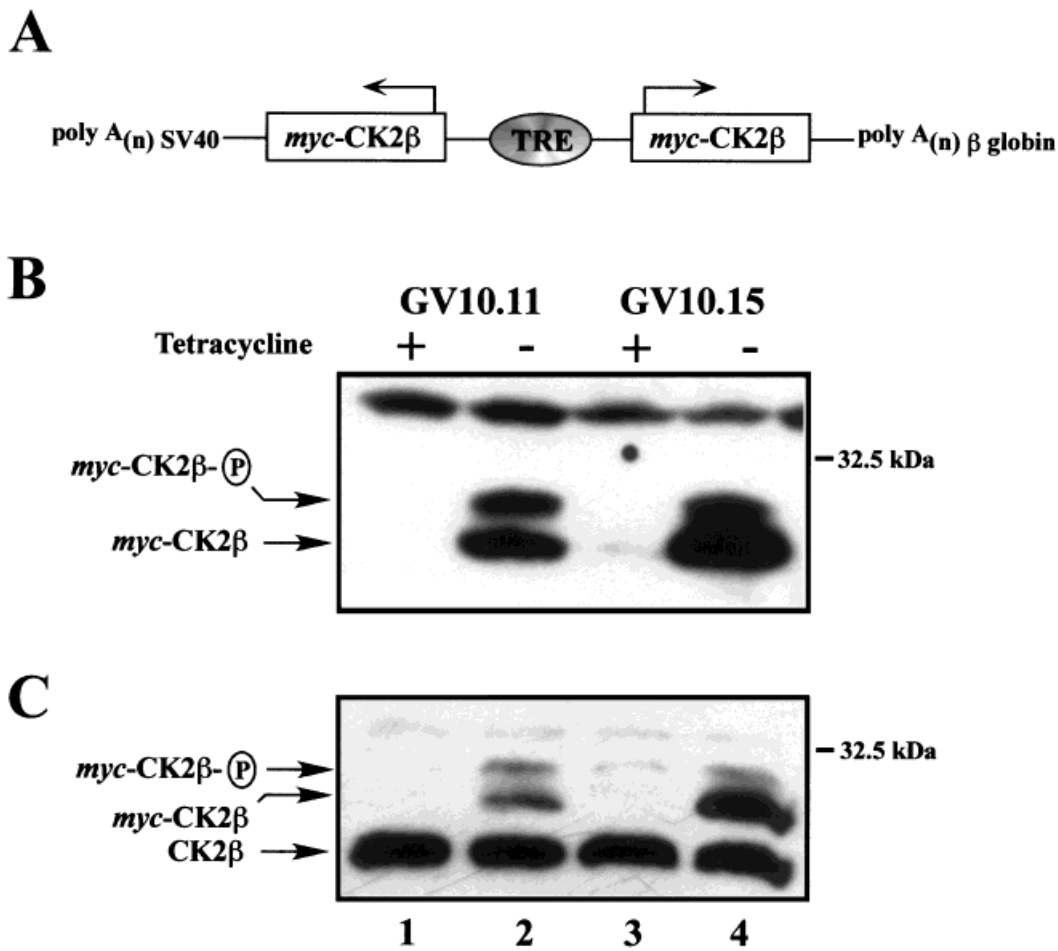

Fig. 1. Inducible expression of *myc*-CK2β in U2OS cells. Human osteosarcoma U2OS cells were stably transfected with (**A**) a tetracycline-regulated bi-directional construct expressing *myc*-tagged CK2β. In the absence of tetracycline, the tetracycline-responsive transactivator protein binds to the tetracycline response elements (TRE) and activates transcription in both directions as shown by the arrows. Osteosarcoma clones, GV10.11 and GV10.15, were seeded in tissue culture dishes with (+) or without (−) tetracycline and allowed to grow for 24 h. Forty micrograms of total lysate was then subjected to SDS–PAGE, transferred to PVDF, and probed with (**B**) anti-*myc* or (**C**) anti-CK2β antibodies. The phosphorylated and non-phosphorylated forms of *myc*-CK2β as well as endogenous CK2β are indicated by the arrows. The 32.5 kDa molecular weight marker is indicated on panels B and C.

the expression of *myc*-CK2β in these U2OS cell lines (Fig. 1B,C, lanes 1 and 3). In the absence of tetracycline, expression of *myc*-CK2β is induced as monitored by Western blotting (Fig. 1B,C, lanes 2 and 4). Both the phosphorylated and non-phosphorylated forms of *myc*-CK2β can be seen in both Western blots (Fig. 1B,C). Treatment of total cell extracts with lambda phosphatase was performed to document that the slower migrating band is indeed the phosphorylated form of *myc*-CK2β (data not shown). GV10.15 cells expressed *myc*-CK2β at levels equivalent to that of endogenous CK2β (Fig. 1C, lane 4) whereas GV10.11 cells express *myc*-CK2β at levels somewhat lower than endogenous CK2β. Since the GV10.15 cell line express *myc*-CK2β at higher levels, this cell line was chosen for further study.

To determine the effect of the increased expression of CK2β on total CK2 kinase activity, we performed kinase assays in whole cell lysates of GV10.15 cells (Fig. 2A). At 48 h following induction of *myc*-CK2β, cell lysates were collected and total CK2 activities were determined using a synthetic CK2 substrate. Expression of *myc*-CK2β in cells resulted in an increase of approximately 1.5-fold in total CK2 activity likely due to the formation of additional tetrameric CK2 complexes with endogenous catalytic subunits (Fig. 2A). Immunofluorescence staining was also performed using antibodies against the *myc* tag of CK2β to examine

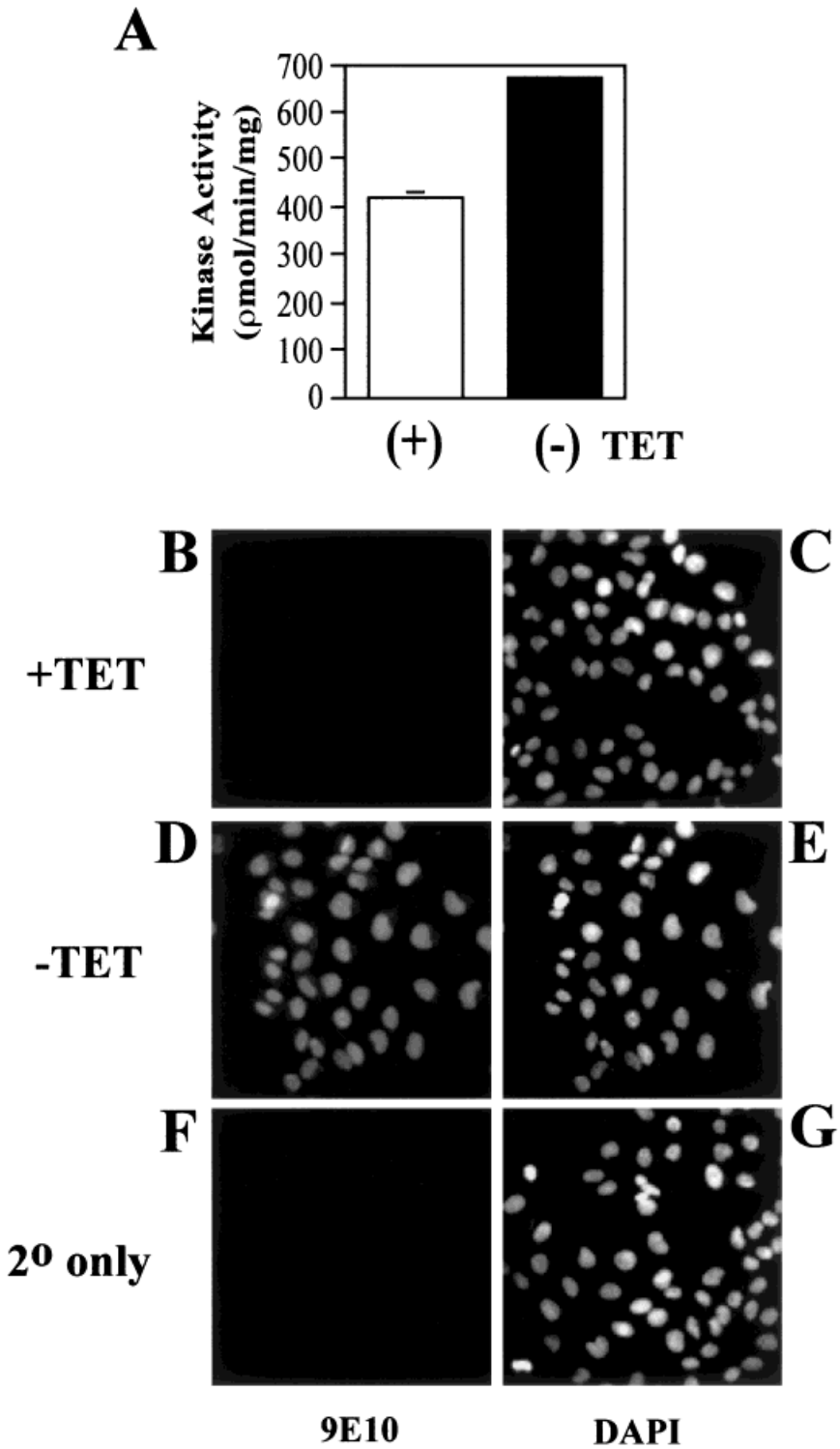

Fig. 2. Kinase activity and immunofluorescent detection of *myc*-CK2β in U2OS cells. GV10.15 cells were seeded in culture dishes or coverslips with (+) or without (−) tetracycline (TET) and induced for expression of *myc*-CK2β. **A:** Total CK2 activity was measured in duplicate following 48 h of induction in total cell extracts using [γ-^{32}P]ATP and RRRDDDSDDD substrate as indicated in the Materials and Methods. **B–G:** Fluorescent examination of cells grown in the presence (B, C) or absence (D–G) of tetracycline. At 24 h post induction, GV10.15 cells that were grown on coverslips in the presence or absence of tetracycline were subjected to indirect immunofluorescence using anti-*myc* (9E10) antibodies and a FITC-GAM secondary antibody as described in Materials and Methods (B, D). The same coverslips were counter-stained with DAPI to visualize cellular nuclei (C, E). As a control, GV10.15 cells grown in the absence of tetracycline were incubated with the secondary FITC-GAM antibody in the absence of anti-*myc* 9E10 antibodies (F). The same cells were counter-stained with DAPI (G).

the sub-cellular distribution of *myc*-CK2β and to ensure that *myc*-CK2β was induced homogeneously in the entire cell population. Figure 2B shows GV10.15 cells in the presence of tetracycline and confirms that there is a tight regulation of the expression of *myc*-CK2β in these U2OS clones since anti-*myc* staining is absent. In the absence of tetracycline, *myc*-CK2β localizes mainly to the nucleus with lesser amounts seen in the cytoplasm (Fig. 2D). Importantly, when we compare anti-*myc* staining and the DAPI-stained images of GV10.15 cells in the absence of tetracycline, it is apparent that the entire population responds by expressing *myc*-CK2β (Fig. 2D,E, respectively).

Incorporation of *myc*-CK2β Into Tetrameric CK2 Complexes

Having demonstrated that the cell lines exhibited regulated expression of CK2β, we wanted to determine whether *myc*-CK2β was entering into CK2 complexes containing catalytic CK2 subunits. To this end, we performed immunoprecipitations using anti-CK2α and anti-*myc* antibodies on GV10.15 whole cell lysates derived from cells that were grown in the absence or presence of tetracycline for 48 h. We then performed immunoblots to examine those immunoprecipitates for the presence of either CK2α or *myc*-CK2β. By first probing the blot with anti-*myc* antibodies (Fig. 3A), we noted that both the phosphorylated and non-phosphorylated forms of *myc*-CK2β were present in the anti-*myc* immunoprecipitates (Fig. 3A, lane 6) from extracts prepared from cells grown in the absence of tetracycline. By comparison, *myc*-CK2β was not detected when immunoprecipitates were performed with extracts from cells grown in the presence of tetracycline (Fig. 3A, lane 7). The corresponding CK2α immunoblot indicated that endogenous CK2α was also present in the anti-*myc* immunoprecipitates (Fig. 3B, lane 6) in the absence of tetracycline whereas CK2α was absent in the anti-*myc* immunoprecipitates from GV10.15 cells that were cultured in the presence of tetracycline (Fig. 3B, lane 7). To confirm this interaction between these two CK2 subunits, we performed the reciprocal experiment in which we immunoprecipitated CK2α (Fig. 3B, lanes 3–5) and examined the immunoprecipitates for the presence of *myc*-CK2β. In this situation, we noted that the majority of *myc*-CK2β co-immunoprecipitating with CK2α was in its phosphorylated form (Fig. 3A, lane 3). Since CK2β is only phosphorylated in tetrameric CK2 complexes [Penner et al., 1997], this result indicated that *myc*-tagged CK2β is behaving as a native CK2 regulatory subunit and entering into tetrameric CK2 complexes with endogenous catalytic CK2 subunits.

Cellular Effects of Increased CK2β Expression

Published reports suggest that CK2β has a role in cell cycle progression and proliferation [Lorenz et al., 1993; Li et al., 1999]. Thus, we investigated the role of CK2β in the GV10.15 cells in these processes. To this end, we investigated cell cycle profiles using propidium iodide staining at various times following induction of *myc*-CK2β in GV10.15 cells. For a period of up to 3 days following induction, we harvested GV10.15 cells and stained the cellular DNA with propidium iodide to be analyzed by FACS (Fig. 4A). In contrast to what was previously observed in cells with constitutive expression of *myc*-CK2β [Li et al., 1999], no changes in the percentage of each phase of the cell cycle were observed following induction of *myc*-CK2β in GV10.15 cells. A cell proliferation assay on the GV10.15 cells up to a period of 8 days with or without tetracycline also demonstrated no significant change in proliferation due to the induced expression of *myc*-CK2β. Furthermore, the GV10.15 cells portrayed the same morphological phenotype upon expression of CK2β (data not shown). Again, these results contrast previous observations where dramatic alterations in proliferation or cell cycle profiles resulted from constitutive expression of *myc*-CK2β [Li et al., 1999]. GV10.11, another cell line exhibiting regulated expression of *myc*-CK2β, showed the same results in the proliferation assay as the GV10.15 cells (data not shown). Collectively, these results demonstrate that increased expression of CK2β in U2OS cells does not cause changes in the cell cycle profile or proliferation.

Based on previous work suggesting a role of CK2β in DNA damage pathways [Teitz et al., 1990; Toczyski et al., 1997], we investigated the UV damage response in GV10.15 cells at various time points after exposure to 10 J/m^2 (Fig. 5A) or 40 J/m^2 (Fig. 5B) UVC irradiation. In this experiment, we initially induced *myc*-CK2β in GV10.15 cells for 48 h. After exposure to either fluence of UVC, the cells were subsequently allowed to recover at 37°C in an atmosphere of

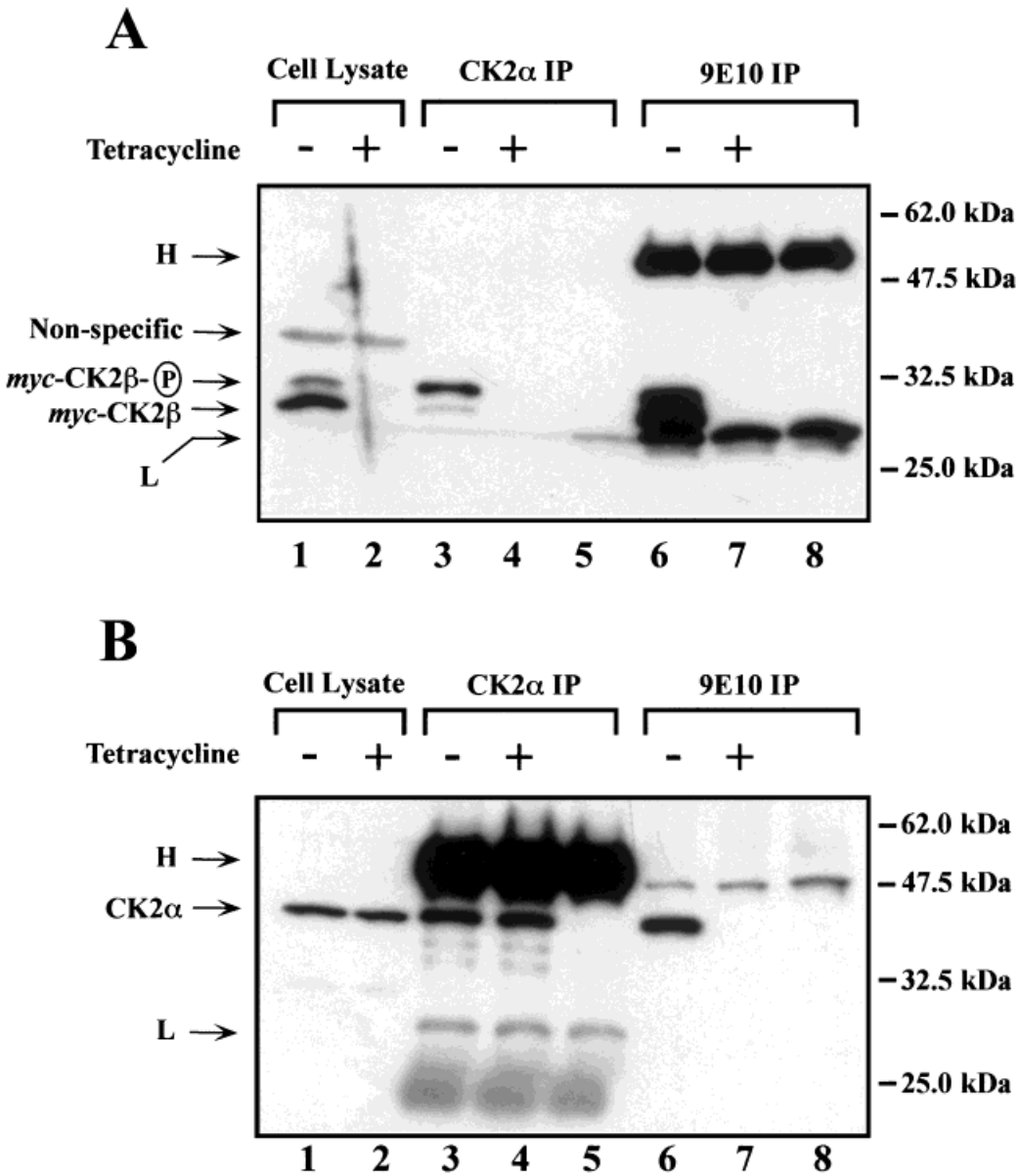

Fig. 3. *Myc*-CK2β enters into CK2 complexes containing endogenous CK2α in GV10.15 cells. GV10.15 cells were seeded in tissue culture dishes with (+) or without (–) tetracycline for 48 h. The cells were then harvested in 1% NP40 lysis buffer. Immunoprecipitations (IP) were performed with antibodies either against CK2α (**lanes 3–5**) or against the *myc* epitope (**lanes 6–8**). As a control, both anti-CK2α and anti-*myc* antibodies (9E10) were incubated with lysis buffer only (**lanes 5** and **8**, respectively). Whole cell lysates (**lanes 1** and **2**) and immunoprecipitates (**lanes 3–8**) were subjected to 12% SDS–PAGE. Immunoblots were probed with (**A**) anti-*myc* (9E10) antibodies or (**B**) anti-CK2α antibodies. The molecular weight markers are shown on the right and *myc*-CK2β and CK2α are marked on the left. The Heavy (H) and Light (L) chains of IgG as well as a non-specific band are also indicated.

5% CO_2. The irradiated GV10.15 cells were then lysed in sample buffer at the indicated time points and analysed by Western blotting with either anti-p53 (DO1) or anti-*myc* (9E10) antibodies to examine the p53 and *myc*-CK2β at various times following UV treatment. In both the 10 J/m^2 (Fig. 5A) and the 40 J/m^2 (Fig. 5B) UVC irradiated samples, the total p53 profiles were identical in the presence or absence of tetracycline at each time point suggesting that increased expression of CK2β does not affect p53 induction. When we followed the expression pattern of *myc*-CK2β between the two irradiated samples, we noted different results. When cells were exposed to 10 J/m^2 UVC, levels of both the phosphorylated (slower migrating)

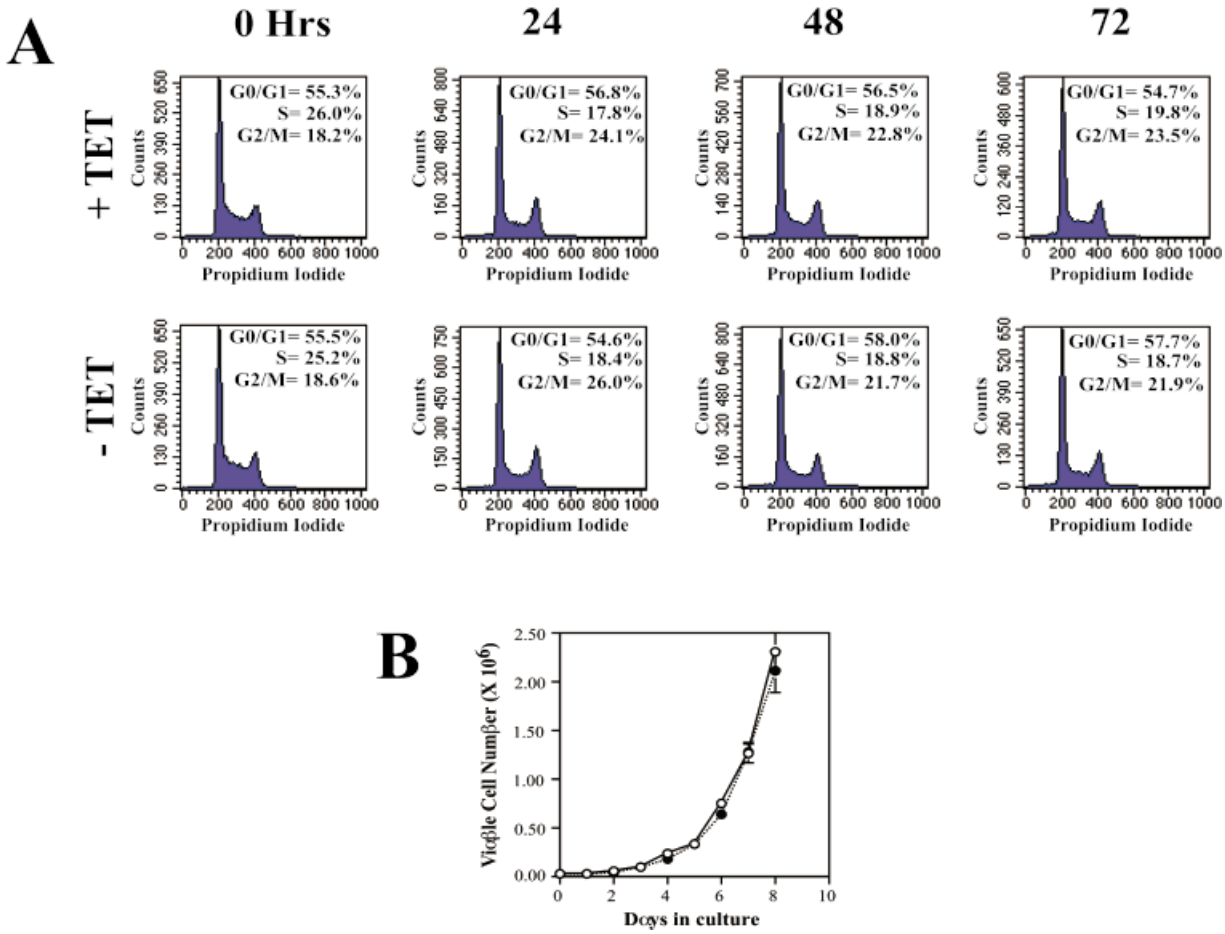

Fig. 4. Cell cycle profiles and cell proliferation analysis of GV10.15 cells. **A:** Human osteosarcoma GV10.15 cells were seeded in 10-cm dishes in the presence of tetracycline. On the next day (Time = 0 h), the cells were extensively washed with PBS and the media was replaced with (+TET) or without tetracycline (−TET). At 24-h time intervals, cells were collected, fixed in 95% ethanol, stained with propidium iodide, and analyzed by FACS. The percentage of cells present in each phase of the cell cycle is indicated at each time point. **B:** GV10.15 cells were seeded in 6-well dishes in the presence of tetracycline. On the next day (Time = 0 day), the cells were grown with (open circles) or without (closed circles) tetracycline. At 24-h intervals up to a period of 8 days, the GV10.15 cells were collected, as described in the Materials and Methods, and counted in triplicate using a hemocytometer. The error bars indicate standard deviation at each time point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and the non-phosphorylated (faster migrating) forms of *myc*-CK2β decreased at 8 h after UV exposure. At subsequent time points, expression of both forms of *myc*-CK2β was restored to the same levels as was seen prior to UV exposure. However, the situation was different in the 40 J/m^2 irradiated GV10.15 cell sample in that the non-phosphorylated form of *myc*-CK2β exhibited a complete disappearance at 8 h following UV exposure (Fig. 5B) with the slower-migrating phosphorylated form of *myc*-CK2β exhibiting a complete disappearance 48 h after UV exposure. These results probably reflects the fact that the phosphorylated form of *myc*-CK2β has a longer half-life since it has entered into native CK2 tetrameric complexes where it is more stable than uncomplexed CK2β subunits. In contrast to the results seen with the lower dose of UV, the loss of *myc*-CK2β expression that was seen following exposure of 40 J/m^2 UVC was not restored.

The original objective of this study was to systematically examine cell lines with regulated expression of CK2β in order to build upon the previous observation that overexpression of CK2β attenuates cell proliferation [Li et al., 1999]. However, no effects due to increased expression of CK2β were observed in either the GV10.15 or GV10.11 cells. Since observed changes in cell proliferation upon expression of CK2β were previously observed in mouse fibroblast 3T3-L1 cells, we were concerned that

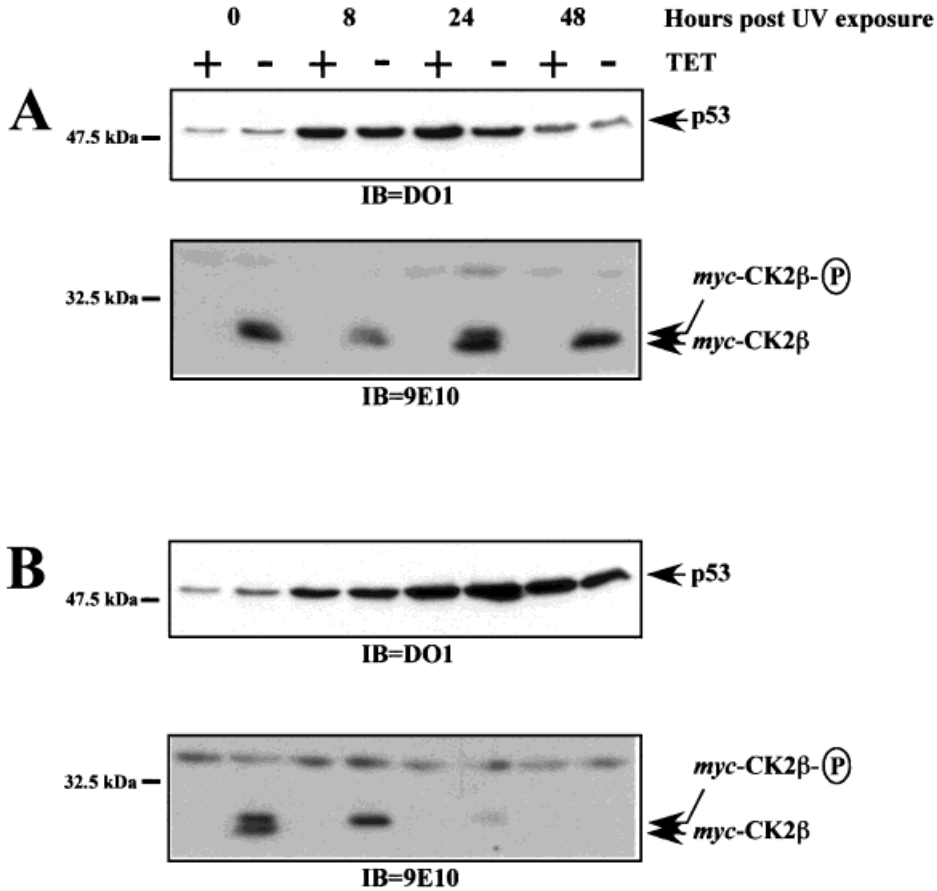

Fig. 5. Examination of p53 and *myc*-CK2β levels in GV10.15 cells exposed to ultraviolet radiation. GV10.15 cells were seeded in 10-cm dishes and grown for 48 h in the presence of tetracycline (+) or in the absence of tetracycline (−) to induce expression of CK2β. These cells were then exposed to either (**A**) 10 J/m^2 or (**B**) 40 J/m^2 UVC irradiation as described in the Materials and Methods. Following irradiation, GV10.15 cells were harvested in lysis buffer at the indicated time points. Extracts were subjected to SDS–PAGE, and analysed on immunoblots (IB) with either anti-p53 (DO1) or anti-*myc* (9E10) antibodies as indicated. Bands corresponding to p53 and the phosphorylated and non-phosphorylated forms of *myc*-CK2β are indicated. Molecular weight markers are also indicated.

the failure of *myc*-CK2β to induce altered cell proliferation in the present study may have been a unique feature of U2OS cells. To address this issue, we made efforts to generate tetracycline-inducible mouse 3T3-L1 cells that express the *myc*-tagged CK2β using the same bi-directional promoter that had been used to generate the U2OS cell lines. From a total number of forty-five drug-resistant clones, we obtained three clones, designated RD1, RD3, and RD4, which expressed *myc*-CK2β at levels that could be readily detected in total cell lysates. We were unable to obtain tightly regulated expression of *myc*-CK2β in any of these cell lines. Nevertheless these cell lines exhibited *myc*-CK2β expression apparently exceeding that seen previously [Li et al., 1999] since *myc*-CK2β was readily detected by immunoblot analysis of cell extracts with anti-*myc* antibodies (Fig. 6A). Therefore, we used these cell lines to study the effect of increased expression of CK2β on 3T3-L1 cells. Levels of *myc*-CK2β were also compared to levels of endogenous CK2β using immunoblots detected with anti-CK2β (Fig. 6B). Although *myc*-CK2β levels are clearly lower than endogenous CK2β, those results again suggest that *myc*-CK2β levels in these cells exceed the levels previously seen [Li et al., 1999] where *myc*-CK2β was undetectable in whole cell lysates. The same experiment was performed in the presence of tetracycline with identical results (data not shown). We then investigated whether the expression of *myc*-CK2β influenced the proliferation of each of the mouse 3T3-L1 clones. To this end, we seeded RD1, RD3, and RD41 clones as well as the parental cell line in medium without tetracycline and counted the cells daily

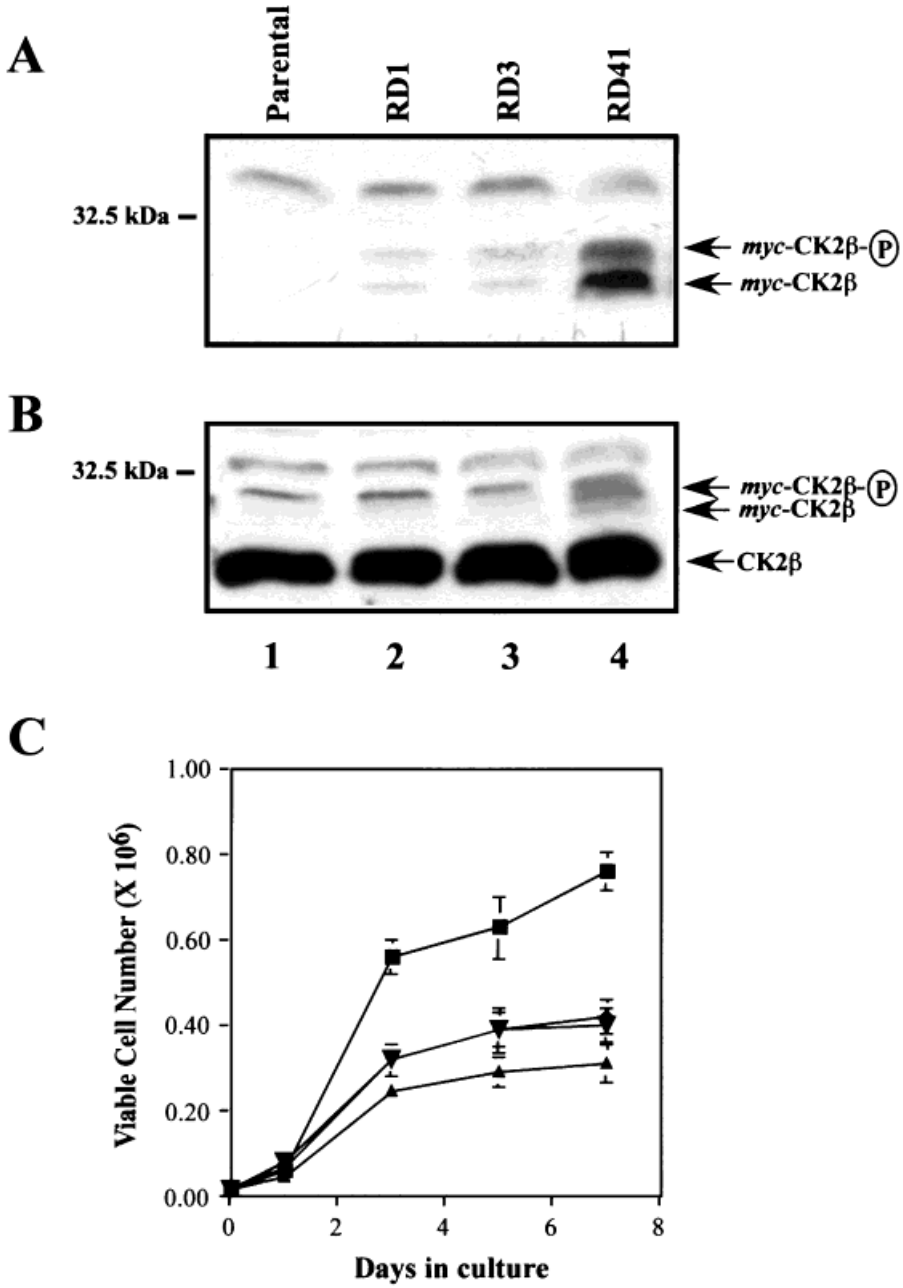

Fig. 6. Examination of proliferation in mouse 3T3-L1 cells stably expressing *myc*-CK2β. **A,B:** Whole cell lysates from three 3T3-L1 clones as well as the parental 3T3-L1 cell line grown in the absence of tetracycline for 24 h were subjected to 12% SDS–PAGE and analyzed on immunoblots using anti-*myc* (9E10) (panel A) and anti-CK2β antibodies (panel B), respectively. The various forms of CK2β as well as the molecular weight markers are indicated. **C:** These mouse 3T3-L1 stable cell lines were initially seeded in tissue culture dishes in the presence of tetracycline. At time 0, the tetracycline was removed. Cells were counted on a hemocytometer daily for a period of 8 days in triplicate. The error bars indicate SD at each time point. The symbols used are: (■) RD1, (▲) RD3, (▼) RD41, and (◆) parental 3T3-L1 cells.

for a period of 8 days. Expression of *myc*-CK2β in the 3T3-L1 clones did not have any effect on the proliferation of these cells nor were there any observable morphological changes in these cells in comparison to the parental cell line (Fig. 6C, data not shown). Importantly, if there were an effect of cellular proliferation due to altered expression of CK2β, we would expect a graded response with the RD41 clone showing the greatest effect on proliferation and the RD1 clone showing the least effect. As seen in Fig. 6C, there was no correlation between levels of

myc-CK2β and proliferation rate suggesting that, as in the U2OS cells, altered expression of CK2β does not result in attenuated proliferation. The same experiment was performed in the presence of tetracycline with identical results (data not shown).

DISCUSSION

Mounting evidence suggests that CK2β is capable of functions distinct from its roles with the catalytic CK2 subunits in the tetrameric holoenzyme complex. For example, studies in yeast indicate that CK2β plays a role in cell growth and cytokinesis and in an adaptation checkpoint that is triggered by DNA damage [Roussou and Draetta, 1994; Toczyski et al., 1997]. In vertebrates, the CK2 regulatory subunit is also suggested to participate in cell cycle progression, cellular proliferation, as well as radiation-induced DNA damage signaling [Lorenz et al., 1993; Toczyski et al., 1997; Li et al., 1999]. At the molecular level, free CK2β can interact with the p53 tumor suppressor and is presumed to direct some of its cellular processes [Filhol et al., 1992; Appel et al., 1995; Gotz et al., 1996; Prowald et al., 1997; Schuster et al., 1999]. Other CK2β-interacting proteins that appear to interact with CK2β in the absence of catalytic CK2 subunits include A-Raf and c-Mos [Boldyreff and Issinger, 1997; Chen and Cooper, 1997; Chen et al., 1997; Hagemann et al., 1997; Li et al., 1997]. In the case of the latter, CK2β inhibits the protein kinase activity of c-Mos in a manner such that overproduction of CK2β in *Xenopus* oocytes delays meiotic maturation. A recent study demonstrated that transient or stable constitutive overexpression of CK2β resulted in cell cycle perturbations and attenuated cell proliferation [Li et al., 1999]. Despite the accumulation of evidence involving CK2β in these cellular processes, the molecular pathways that involve CK2β in mammalian cells remain poorly defined. To address this issue, we developed tetracycline-inducible cell lines that provide an opportunity for a systematic investigation of the mechanisms by which CK2β regulates cellular events.

To develop these stably transfected cell lines, we attached a single *myc* epitope tag to the expressed form of CK2β in order to distinguish it from endogenous CK2β. Immunofluorescence localization experiments as well as immunoprecipitation and immunoblotting experiments confirmed that *myc*-CK2β localizes to the nucleus and forms complexes with catalytic CK2 subunits. Also, the majority of *myc*-CK2β that associated with CK2α was phosphorylated as indicated by the mobility shifts on Western blots. The increased expression of CK2β resulted in a significant increase in the cellular levels of CK2 activity, but did not effect any changes in cell cycle profiles or proliferation. Moreover, our results do not appear to be unique to U2OS cells since altered expression of CK2β did not have an effect on proliferation in stably transfected 3T3-L1 cells.

It is intriguing that elevated expression of CK2β has been observed in a number of tumors or cell lines including colorectal carcinomas, head and neck tumors, and in prostate [Munstermann et al., 1990; Stalter et al., 1994; Yenice et al., 1994; Faust et al., 1996]. Furthermore, between normal and tumor cells, the ratio of catalytic to regulatory CK2 subunit was observed to be altered [Stalter et al., 1994]. By altering the expression of CK2β in U2OS and 3T3-L1 cells, we may have been mimicking the situation seen in some of these tumors. Although we cannot exclude the possibility that altered expression of CK2β can affect proliferation under some circumstances, our results suggest that a change in the expression of CK2β is not by itself sufficient to induce alterations in cell proliferation that are frequently observed in tumors or cancer-derived cells.

Our conclusions are not in agreement with previous published data [Li et al., 1999]. In that study, 3T3-L1 cell lines were established that constitutively express CK2β that had been modified by the addition of six copies of the *myc* epitope tag (i.e., 6X-*myc*-CK2β). The fact that this 6X-*myc*-tagged CK2β could only be detected in immunoprecipitates and could not be detected in whole cell lysates suggests that the overall levels of 6X-*myc*-CK2β expression were relatively low. Furthermore, since this low level expression was under the control of an unregulated promoter, it is difficult to conclude that the degree of inhibition of cell proliferation results from increased CK2β rather than some other event that occurred during the generation of the stably transfected cell lines. In addition, the use of a large *myc* epitope tag may have changed the characteristics of the expressed CK2β such that the tag rather than CK2β was responsible for the dramatic alterations in

proliferation and cell cycle profiles. Although we cannot exclude the possibility that a single *myc* epitope may have perturbed the functions of CK2β, we do not observe any features of *myc*-CK2β that distinguish it from native CK2β. Furthermore, we believe that a single epitope would be less likely to perturb CK2β functions than would multiple copies of the epitope. Even in the stably transfected 3T3-L1 that we have utilized in this present study, we could readily detect *myc*-CK2β in whole cell extracts suggesting that levels of *myc*-CK2β exceeded the levels seen in the cell lines generated by Li et al. [1999]. In the three independent 3T3-L1 cell lines that we have characterized in this study, we observed no correlation between levels of *myc*-CK2β and proliferation rate. In fact, two of these cell lines exhibited proliferation rates nearly identical to that observed with the parental 3T3-L1 cells.

Another possible difference between our study and that of Li et al. [1999] resides in the different experimental strategies utilized to achieve overexpression of CK2β. In order to achieve regulated expression and to minimize issues of clonal variation, we utilized a system with tetracycline-regulated expression of CK2β. This system has been widely used to achieve the regulated expression of numerous different constructs in cells and in animals. However, since this system involves the expression of a tetracycline-regulated transcriptional activator fusion protein, it is conceivable that this tetracycline-regulated transcriptional activator fusion protein may affect the functions of other cellular proteins. In this vein, if the tetracycline-regulated transcriptional activator directly, or indirectly, influenced CK2β, it is possible that it could mask the cellular effects of CK2β overexpression. Although we cannot directly exclude this possibility, it is noteworthy that the recent report of Lebrin et al. [2001], who did not utilize an inducible expression system, also demonstrated that overexpression of CK2β is without effect on cell proliferation. Overall, we do not have a precise explanation for the discrepancy between our results or those of Lebrin et al. [2001] and those previously reported [Li et al., 1999]. At least in part, this discrepancy may reflect the complex cellular functions of CK2β. Moreover, we are confident that the use of an inducible expression system overcomes many of the limitations such as issues of clonal variation that are associated with constitutive overexpression.

In light of previous evidence suggesting that CK2β is involved in DNA damage signaling and that CK2β interacts with cell cycle checkpoint proteins [Teitz et al., 1990; Toczyski et al., 1997], we investigated the effects of altered CK2β expression on cellular responses to UV light. In contrast to what had been observed in Xeroderma Pigmentosum cells, we did not observe any protective effect of increased CK2β expression when the U2OS cells were UV-irradiated. Direct observation showed no change in morphology when comparing cells in the absence or presence of tetracycline (data not shown). Furthermore, levels of p53 induction following UV exposure were unaffected by alterations in the expression of CK2β. As was the case with the effects of altered CK2β expression on proliferation, our results indicate that alterations in CK2β are not sufficient to alter the ability of all cells to respond to UV treatment.

It is also noteworthy that the expression levels of *myc*-CK2β were affected to different degrees by different doses of UV. At the lower dose of UV, *myc*-CK2β exhibited a transient decrease in levels at 8 h but *myc*-CK2β levels were subsequently restored to levels seen prior to UV treatment. By comparison, at the higher does of UV, levels of *myc*-CK2β failed to recover. In the latter case, the non-phosphorylated form of *myc*-CK2β appeared to disappear at a faster rate than the phosphorylated *myc*-CK2β likely because the phosphorylated form is present in long-lived CK2 holoenzyme complexes. The difference between *myc*-CK2β levels between low and high doses of UV could be due to the degree of DNA damage inflicted on the cell. At low UV doses, global transcription would be only transiently inhibited whereas at high UV doses, global transcription would cease for a long period to allow for DNA repair.

In summary, there is a substantial body of literature that suggests that CK2β has important roles in various aspects of cellular regulation. Moreover, there is justifiable cause to expect that alterations in the expression of CK2β will profoundly affect cell behavior. However, using the model system described in this paper to systematically alter the cellular levels of CK2β, we have not observed any alterations in cell proliferation, cell cycle profiles or responses to UV that result from changes in

CK2β. The differences between our results and those from previous reports further emphasize the complex nature of CK2β and its cellular functions. Furthermore, our results indicate that increased expression of CK2β is not by itself universally sufficient to effect alterations in cell proliferation or responses to DNA damage.

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