Development of a Stabilized Form of the Regulatory CK2β Subunit That Inhibits Cell Proliferation

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A number of cancers are characterized by elevated expression of CK2 (formerly casein kinase II), which has been implicated as a key component in cell proliferation and transformation. Two lines of evidence, (a) deregulated expression of CK2 and (b) CK2β ubiquitination and degradation of these in a proteasome-dependent manner prompted further investigation of the regulation of CK2β protein stability. We demonstrate that mutating six surface-exposed lysine residues to arginine (6KR) to interfere with ubiquitin attachment can stabilize CK2β. Examination of 6KR expression in cells revealed increased stability over time and increased its steady-state expression level compared with CK2β. In cells, 6KR was no longer sensitive to proteasome inhibition but maintained an elevated expression level. In our studies, 6KR functioned as a normal CK2 regulatory subunit, because it participated in CK2β dimerization, associated with catalytic subunits, was autophosphorylated, and formed active, stable CK2 tetramers. The physiological role of CK2β stabilization was investigated in cell proliferation assays, which showed a significant decrease in proliferation in cells expressing 6KR compared with CK2β. Overall, our results indicate that a stabilized form of CK2β can be used to inhibit cell proliferation.

Fundamental cellular processes such as proliferation and survival involve regulation by CK2 (formerly casein kinase II),3 a serine/threonine protein kinase that is ubiquitously expressed in eukaryotic cells (1). Further evidence for its critical role is revealed in the absolute requirement for CK2 for viability in yeast and slime mold and in the requirements for CK2 in the G1/S and G2/M cell cycle transitions in yeast and mammalian cells (2–7).

Given the diverse, yet essential, roles of CK2 within the cell, it is important to understand the mechanisms regulating CK2, which are equally as diverse and critical. Furthermore, perturbations in expression or activity of CK2 are associated with human disease. Abnormally high levels of CK2 have been observed in cancers of the breast, prostate, lung, head and neck, and kidney (8–12). Overexpression of catalytic CK2 subunits led to increased proliferation and transformation. By comparison, overexpression of the regulatory CK2 subunit has been associated with decreased proliferation in yeast and mammalian cells, although this inhibitory role has not been universally observed (13–15). Collectively, these results indicate that CK2 has a profound effect on cell proliferation and suggests that individual CK2 subunits may exert competing effects.

CK2 has typically been viewed as a tetrameric complex consisting of two catalytic subunits, CK2α and CK2α', and one regulatory subunit, CK2β (16–20). Studies investigating CK2 tetramer assembly determined that a dimer of CK2β subunits forms the core of the enzyme, and the catalytic subunits subsequently bind to the CK2β core (21–26).

CK2β is phosphorylated at serine 209 in a cell-cycle-dependent manner by p34cdc2 in vitro and in mammalian cells (27–30). CK2β autophosphorylation at serines 2 and 3 is mediated by the catalytic subunits of CK2 (28, 31). Although neither phosphorylation event is completely understood, there are indications that autophosphorylation of CK2β enhances its stability (27). Detailed investigation of CK2β protein stability and turnover revealed that CK2β exhibits a biphasic degradation pattern (32). More specifically, CK2β is normally expressed at a higher level than the catalytic subunits of CK2, allowing some CK2β to be incorporated into CK2 tetramers and stabilized, whereas the excess CK2β is rapidly degraded with a half-life of less than 1 h (32). Furthermore, the observed ubiquitination of CK2β and the accumulation of CK2β protein upon proteasome inhibition suggested that polyubiquitination of CK2β targets it for degradation (27).

We hypothesized that the stability of CK2β could be altered by mutations that disrupt its ubiquitination. Alterations in CK2β protein levels may influence the mechanisms that govern the affect of CK2 on cell proliferation, which may be important in understanding the onset of cancer. In this report we successfully generated a form of CK2β, designated 6KR, that exhibits altered stability in cells. Further studies characterize the 6KR protein and examine its effect on cell proliferation.
Plasmid Constructs—The previously described (24) HA-CKβ construct was inserted into the HindIII/XhoI sites of the pcDNA3.1(+) plasmid (Invitrogen). Site-directed mutagenesis was used to individually mutate the nine lysine residues of CKβ (amino acids 33, 100, 134, 139, 147, 177, 191, 208, and 212) to arginine, creating a set of HA-tagged single lysine mutants of CKβ. The following primers were used: 5'-CCAGGACAGTTTAACTTTAC-3' (K33R-sense), 5'-AGAGTTTAAATCTGTCCGGATGAT-3' (K33R-antisense), 5'-ATGGTGGAAGATGACGAGCAG-3' (K100R-sense), 5'-CTGGTACCTTTCCAAATCGGT-3' (K100R-antisense), 5'-CCATGGTTGAGCTCTACTG-3' (K134R-sense), 5'-GCAGTAGGCCTCACCATGG-3' (K134R-antisense), 5'-ACTGCCACAGGTGACATG-3' (K139R-sense), 5'-TCCATGCACCTGGGGCAG-3' (K139R-antisense), 5'-CACACCGAGTTCAAGG-3' (K147R-sense), 5'-TCTGATGCCTGATTGGTGT-3' (K147R-antisense), 5'-ACGGCCCAAGGAGACCTG-3' (K177R-sense), 5'-GCAGGATCGCTCTGCGG-3' (K177R-antisense), 5'-CGGTTCAGGATCATCCCG-3' (K191R-sense), 5'-GGATGGATCTGCTGAAACCG-3' (K191-antisense), 5'-GCAATTTTCAAGGCGCCAG-3' (K208R-sense), 5'-GACCTGGCCCTGATGAGT-3' (K208R-antisense), 5'-CCCCAGGAGCACCATGC-3' (K212R-sense), and 5'-CGAATCTCCGACTGGG-3' (K212R-antisense). Double and multiple lysine to arginine mutations were introduced into HA-CKβ sequentially, using the appropriate primers to generate HA-K177R/K139R and HA-K177R/K191R, HA-5KR, HA-6KR, and HA-9KR (Fig. 1C).

Plasmids encoding N-terminal His-tagged CKβ or 6KR were constructed by inserting CKβ or 6KR DNA into the NotI/Apal sites located 3’ to the 6×-histidine tag in pcDNA3.1(+) (His-CKβ and His-6KR, respectively). Myc-6KR was constructed by inserting Myc-tagged 6KR DNA into the MluI and Nhel sites of the pRe/CMV vector. Full-length Myc-CKβ was described previously (33). pMT107, a plasmid encoding His-tagged ubiquitin was obtained from D. Bohmann. Full-length CK2α and CK2α’ were cloned into pGEX-3X and pEGFP-C2 using BamHI, as described previously (34). pBABEpu6 encodes a puromycin N-acetyltransferase was obtained from G. Evan. Bidirectional plasmids encoding both CK2α-HA and Myc-CK2β or Myc-6KR were constructed by inserting CK2α-HA into the PstI sites and inserting Myc-CK2β or Myc-6KR into the MluI/Nhel sites of the tetracycline-regulated vector pBl (Clontech). All constructs were verified by DNA sequencing.

Antibodies—Polyclonal antibodies raised against CKβ, CK2α, and CK2α’ have been previously described (28, 35). Monoclonal antibodies directed against the HA epitope (12CA5) and the biotinylated anti-HA (3F10) were purchased from Roche Applied Science. Monoclonal biotinylated anti-Myc (9E10) was purchased from Sigma. Polyclonal antibodies directed against green fluorescent protein (GFP) were purchased from Molecular Probes. Anti-β-tubulin antibodies were a generous gift from Lina Dagnino (Dept. of Physiology and Pharmacology, University of Western Ontario). Goat-anti-rabbit and Goat-anti-mouse secondary antibodies conjugated to horseradish peroxidase or alkaline phosphatase were purchased from Bio-Rad. Monoclonal anti-biotin secondary antibody conjugated to horseradish peroxidase or alkaline phosphatase was purchased from Jackson ImmunoResearch.

Cell Culture and Transfections—COS7 (green monkey kidney) and HeLa (human cervical cancer) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at 37 °C in an atmosphere of 5% CO2. Cells were transiently transfected using the calcium phosphate precipitation method described previously (33). 16–18 h after transfection, cells were washed and supplied with fresh medium. 24–48 h later cells were harvested in cell lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). Lysates were prepared and used directly or stored at −80 °C.

SDS-PAGE and Immunoblotting—Equivalent amounts of total cellular protein were separated by SDS-PAGE using the method of Laemmli (36). Proteins were transferred to polyvinylidene difluoride, and immunoblots were performed using the following primary antibody dilutions: anti-CKβ (1:2000), anti-CKα (1:1000), biotinylated anti-HA (3F10) (1:500), anti-GFP (1:2000), and anti-β-tubulin (1:100).

Degradation Assays and Proteasome Inhibition Assays—Cells were co-transfected with HA-CKβ or HA-6KR and EGFP-C2 (transfection efficiency marker) as described above. After the DNA precipitate was washed from the cells, each plate of cells was used to seed the appropriate number of new plates so that protein levels could be compared under equivalent transfection efficiencies and allowed to recover for 24 h. For degradation assays, 10-cm plates of cells were treated with 95% ethanol (carrier) or with 150 μg/ml cycloheximide, to inhibit protein synthesis. At various time intervals ranging from 0 to 3 h, cells were harvested and cell lysates prepared. For proteasome inhibition assays, cells were treated with Me6SO (carrier) or with final concentrations of N-carbobenzyoxyl-Leu-Leu-Leuinal (MG132), a proteasome inhibitor, ranging from 0.1 μg/ml (1 μM) to 10 μg/ml (20 μM). After 5 h, cells were harvested and cell lysates were prepared. Total protein concentrations of each lysate were determined, and 30 μg of total protein was analyzed by SDS-PAGE and immunoblotting.

Immunoprecipitations and Binding Assays—Immunoprecipitations and binding assays were performed by transiently transfecting cells as indicated and preparing cell lysates as described above. Immunoprecipitations were preformed on equivalent amounts of total protein using protein-A-Sepharose and anti-HA(12CA5), anti-CKβ, anti-CKα, or anti-CKα’ antibodies as indicated and incubated for 1 h at 4 °C with rotation. After washing, beads were used for CK2 kinase assays or proteins were eluted from the beads. Eluted proteins were analyzed by immunoblot as indicated.

For binding assays cell lysates with equivalent amounts of total protein were incubated with 100 μl of nickel-Sepharose bead slurry (prepared according to the manufacturer’s instructions) for 1 h at room temperature with rotation to specifically isolate histidine-tagged CKβ proteins. Nickel-Sepharose beads were collected, washed twice with 6 ml guanidine-HCl, 12 mM imidazole, 0.1 M sodium phosphate, pH 8.0, twice with 8 M

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urea, 10 mM imidazole, 0.1 M sodium phosphate, pH 6.3, and twice with 50 mM sodium phosphate, pH 8.0, 100 mM KCl, 20% glycerol, 0.2% Nonidet P-40, 7 mM imidazole. Proteins were eluted by incubating the beads with 35 µl of 2× Laemmli sample buffer for 5 min. Eluted proteins were analyzed by immunoblot as indicated.

For ubiquitination experiments, lysates were prepared as described above in urea buffer (8 M urea, 0.1 M NaPO₄, pH 8.0, 10 mM imidazole), and ubiquitinated complexes were isolated by incubating 1500 µg of total protein with 40 µl of Talon resin (BD Biosciences), equilibrated in urea buffer, for 2 h at 4 °C. The Talon resin was washed thoroughly with urea buffer, and bound proteins were eluted by boiling with 40 µl of 2× Laemmli sample buffer. Eluted proteins were analyzed by immunoblot as indicated.

Protein Purification—Bacterially expressed, catalytically active GST-CK2α protein was produced using standard GST fusion protein purification methods. Briefly, BL21(DE3) Escherichia coli-expressing GST-CK2α or GST-CK2α′ cultures were grown, and protein production was induced with 1 mM isopropl-1-thio-β-D-galactopyranoside. Bacterial lysates were prepared and incubated with glutathione-Sepharose at 4 °C with rotation for at least 1 h. Unbound proteins were removed by thorough washing, and GST-CK2α and GST-CK2α′ proteins were eluted with 10 mM or 30 mM reduced glutathione. Fractions containing the highest amounts of GST-CK2α or GST-CK2α′ protein (as determined by SDS-PAGE analysis) were combined, dialyzed into CK2 dialysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 50% glycerol), aliquoted and stored at −20 °C.

CK2 Kinase Assays—Immunocomplexes were prepared as described above. CK2 kinase assays were performed using enzyme immobilized on protein-A-Sepharose beads in 100 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 150 mM NaCl, 0.1 mM ATP (specific activity 500–1000 cpn/pm), in a total volume of 30 µl. The reaction was initiated by adding 2 mM α-casein to each sample. Control reactions contained 2 mM α-casein and were initiated by adding 2 µg of GST-CK2α′. Reactions were incubated at 30 °C for 20 min and stopped by adding 30 µl of 2× Laemmli sample buffer. Samples were boiled and separated by SDS-PAGE, and dried gels were exposed to phosphor screens overnight and scanned. Incorporation of ³²P into α-casein was analyzed, which is directly proportional to the catalytic activity exhibited by the immunoprecipitated CK2 complexes.

Flow Cytometry—For cell cycle analysis, HeLa cells were transfected as described above with HA-CK2β or HA-6KR and EGFP-C2. At the indicated times (ranging from 1 to 5 days) cells were collected using PBS containing 5 mM EDTA, resuspended in PBS containing 2 mM EDTA, resuspended in PBS, filtered using a 40-µm nylon filter, fixed in 1% paraformaldehyde, washed with PBS and permeabilized by dropwise addition into cold 70% ethanol and stored at −20 °C until FACS analysis. Cells were stained with propidium iodide (50 µg/ml), 0.1% sodium citrate, 0.1% Triton X-100, 0.1 mg/ml DNase-free RNase A by incubating for 30 min at 37 °C in the dark and filtered again. 50,000 cells were analyzed on a BD Biosciences FACSCalibur cytometer at a flow rate of less than 200 cells/s. Cell cycle profiles were generated using CellQuest data acquisition and FlowJo cytometry analysis software.

Cell Proliferation Assays—HeLa cells maintained in Dulbecco’s modified Eagle’s medium were plated at a density of 2.5 × 10⁵ cells/6-cm plate. Cells were co-transfected, using ExGen500 (MBI Fermentas), with 4.5 µg of p27, HA-CK2β, HA-6KR, or pcDNA3.1(+), and 0.5 µg of pBABEpuro, a puromycin resistance plasmid. Cells transfected only with 5 µg of pcDNA3.1(+) were used as a negative control. Media was changed 6 h after transfection and 3 h later cells were selected for 16 h with 2 µg/ml puromycin. Five days after transfection, plates were washed with PBS and stained in 0.2% (w/v) methylene blue in methanol at room temperature for 20 min followed by washing with ddH₂O. Images of each plate were converted to grayscale using Adobe Photoshop 7.0. The number of gray pixels (defined as pixels whose levels range from 50 to 200) of the total number of pixels per image was used to determine colony density.

³⁵S Labeling—HeLa cells were transiently transfected as described with HA-CK2β, HA-6KR, pBI(CK2α-HA/Myc-CK2β), pBI(CK2α-HA/Myc-6KR), or left untransfected. Cells were maintained in Dulbecco’s modified Eagle’s medium without methionine, and incubated for 15 min at 37 °C in Dulbecco’s modified Eagle’s medium without methionine. Cells were labeled with 200 µCi of [³⁵S]methionine per plate of cells for 18 h at 37 °C. Lysates were prepared in 500 µl of cell lysis buffer, and 150 µg of total protein was used for each immunoprecipitation. Immunoprecipitations were performed as described above except that anti-HA (12CA5) was coupled to the protein-A-Sepharose beads using standard protocols. To visualize labeled proteins, eluted samples were run on an SDS-PAGE gel, fixed in 50% methanol, 10% glacial acetic acid, soaked in Enhance solution (PerkinElmer Life Sciences), soaked in cold ddH₂O, dried and exposed to autoradiography film for 20 h or more.

RESULTS

Analysis of Potential Ubiquitination Sites in CK2β—Previous studies demonstrated that CK2β is ubiquitinated and degraded by the proteasome (27). To further define the mechanisms controlling CK2 regulation, we were interested in determining whether CK2β protein stability could be altered by systematically removing potential ubiquitination sites. Alignment of the primary amino acid sequence of CK2β from various organisms revealed that each of the nine lysine residues present in human CK2β are highly conserved among species (Fig. 1A). Examination of the crystal structure of CK2β identified six lysines (including 33, 139, 177, 191, 208, and 212) that appear to be surface-exposed and prime targets for ubiquitination (Fig. 1B). Of these six lysines, 33 and 177 protruded further from the surface of CK2β than 139 and 191. Lysines 208 and 212, not present in the crystal structure, are likely surface-exposed because they are
located on the extreme C-terminal tail. The remaining three lysines, 100, 134, and 147, are buried within the structure and are likely important for maintaining CK2β protein structure. Based on this analysis, the six surface-exposed lysines in CK2β were individually mutated to arginine to remove potential ubiquitin attachment sites while maintaining overall protein charge. Fig. 1C illustrates the location of each lysine in CK2β with respect to its functional domains. It is important to note that the HA tag used to facilitate detection of CK2β did not contain any additional lysine residues.

Another important consideration is the low site specificity characteristic of the ubiquitination process (37). Based on this knowledge, we hypothesized that removal of multiple surface-accessible lysines and perhaps even all of the lysines may be required to abrogate ubiquitin-dependent degradation of CK2β. To facilitate examination of this possibility a number of other multiple lysine mutants lacking 5, 6, 7, 8, or all 9 lysine residues were created (Fig. 1C).

Comparison of CK2β Single Lysine Mutant Expression and Degradation—To begin examining the importance of each lysine residue in the regulation of CK2β protein levels, the steady-state expression levels of the single lysine mutants were compared. As shown in Fig. 2A, each mutant was expressed at comparable levels to wild-type CK2β, with the exception of HA-K212R, in COS7 cells. No protein was detected in mock transfected (control) or pcDNA3.1(−/+) transfected cells. To ensure equal loading, membranes were stripped and reprobed with anti-CK2β-tubulin as shown in the lower panel (Fig. 2A).

Collectedly, these results suggest that substitu-
tion of a single lysine residue is insufficient to alter CK2β stability by preventing ubiquitin attachment.

Comparison of CK2β Multiple Lysine Mutant Expression and Degradation—Because substitution of single lysines was ineffective in stabilizing CK2β, we investigated the effects of multiple lysine mutations in CK2β on steady-state expression levels. Each of the double lysine mutants, HA-K177R/K139R and HA-K177R/K191R, as well as HA-5KR, HA-6KR, and HA-7KR, exhibited increased steady-state expression compared with wild type in contrast to the higher levels of HA-6KR that significantly lower amount of ubiquitinated HA-6KR compared with HA-CK2β protein accumulated with increasing concentrations of MG132, HA-6KR protein did not, confirming that HA-6KR was not affected by proteasome inhibition (Fig. 3C). Furthermore, increased levels of autophosphorylated HA-6KR compared with HA-CK2β suggested that HA-6KR is readily incorporated into CK2 tetramers and supramolecular complexes where it is subsequently autophosphorylated. This observation could also indicate that HA-6KR is a less desirable protein phosphatase substrate.

To examine the ubiquitination of CK2β and 6KR, cells were transfected with His-CK2β or His-6KR in the presence or absence of HA-ubiquitin (HA-Ub). Lysates were incubated with a metal affinity resin, to isolate His-tagged protein and any covalently bound proteins. No protein was detected in untransfected (not shown), His-CK2β, or His-6KR transfected samples (Fig. 3D, left panel). However, when cells were transfected with both HA-Ub and His-CK2β a set of ubiquitinated His-CK2β bands were detected. Importantly, these bands increase in intensity upon MG132 treatment and are located at molecular weights that correspond to increasingly ubiquitinated forms of CK2β. Co-transfection of His-Ub and HA-6KR resulted in a significantly lower amount of ubiquitinated HA-6KR compared with wild type in contrast to the higher levels of HA-6KR that are consistently observed (Fig. 3D). To perform the reciprocal experiment, cells were transfected with HA-CK2β or HA-6KR in the presence or absence of His-Ub and incubated with metal affinity resin (Fig. 3D, right panel). Again, an increase in ubiquitinated protein upon MG132 treatment was observed for HA-CK2β but not HA-6KR. Taken together, these results suggest that mutation of the six surface-exposed lysines of CK2β compromise the ability of CK2β to be ubiquitinated and degraded.

Examination of 6KR Protein Stabilization Over Time—To evaluate the stability of HA-6KR compared with HA-CK2β over time, COS7 cells transfected with HA-CK2β or HA-6KR were treated with cycloheximide to inhibit all subsequent protein synthesis. Endogenous CK2β did not significantly degrade upon cycloheximide treatment (Fig. 3E), which was expected, because equilibrium between levels of CK2 tetramer and free subunits would have been achieved prior to transfection. HA-CK2β protein significantly degraded after 1 h of cycloheximide treatment, whereas HA-6KR did not degrade even after 3 h, confirming that HA-6KR protein had indeed been stabilized in cells (Fig. 3F). To ensure equal loading, membranes were was representative of a group of stabilized forms of CK2β (HA-5KR, HA-6KR, and HA-7KR), because modification of its six surface-exposed lysines removed the most potential ubiquitination sites but would have the least impact on overall CK2β structure.

Examination of the Effect of Proteasome Inhibition on 6KR—Because HA-6KR protein was stabilized in cells, we hypothesized that it may no longer be ubiquitinated and therefore no longer sensitive to proteasome inhibition. To test this hypothesis, COS7 cells transfected with HA-CK2β or HA-6KR were treated with increasing concentrations of MG132. No exogenously expressed HA-CK2β or HA-6KR protein could be detected in mock transfected (control) or pcDNA3.1(+) (vector) transfected cells. Whereas HA-CK2β protein accumulated with increasing concentrations of MG132, HA-6KR protein did not, confirming that HA-6KR was not affected by proteasome inhibition (Fig. 3C). Furthermore, increased levels of autophosphorylated HA-6KR compared with HA-CK2β suggested that HA-6KR is readily incorporated into CK2 tetramers and supramolecular complexes where it is subsequently autophosphorylated. This observation could also indicate that HA-6KR is a less desirable protein phosphatase substrate.

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FIGURE 2. Single lysine mutations in CK2β do not alter protein stability. A, COS7 cells were transfected with constructs encoding single surface exposed lysine mutants of CK2β, left untransfected (control) or transfected with pcDNA3.1(+) (vector). Cell lysates were prepared and analyzed by immunoblotting with anti-HA (3F10) antibodies. Positions of HA-tagged CK2β protein and autophosphorylated HA-tagged CK2β protein are indicated. Equal loading was demonstrated by the β-tubulin immunoblot in the lower panel. Results shown are representative of three independent experiments. B, COS7 cells were transiently transfected with the appropriate DNA as indicated. Following transfection, each plate of cells was split into two new plates and treated with Me2SO carrier (−) or with MG132 (+) for 5 h to inhibit the proteasome. Protein levels were analyzed by immunoblot using anti-HA (3F10) antibodies. Results shown are representative of two independent experiments.
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**FIGURE 3. Mutation of multiple lysine residues in CK2β alters protein stability.** A, COS7 cells were left untransfected (control) or transiently transfected with pcDNA3.1(+)(vector) or with plasmids encoding each of the multiple lysine mutants of CK2β. Cell lysates were prepared and analyzed by immunoblotting using anti-HA (3F10) antibodies. Positions of HA-CK2β and autophosphorylated HA-CK2β are indicated. Equal protein loading is demonstrated using β-tubulin stripped and reprobed with anti-β-tubulin as shown in the lower panel (Fig. 3E).

Characterization of 6KR as a Regulatory Subunit of CK2—To assess the integrity of 6KR, we examined its ability to form complexes with other CK2 subunits. Using metal affinity binding experiments the ability of HA-6KR to bind CK2β was addressed. No protein was detected in untransfected, or cells transfected with HA-6KR, His-CK2β, or His-6KR (Fig. 4A). When both His-CK2β or His-6KR and HA-CK2β were present binding was detected. Efficient affinity purification of His-tagged CK2β or 6KR was demonstrated using anti-CK2β antibodies (lower panel). The ability of 6KR to bind another 6KR molecule was examined by immunoprecipitating HA-CK2β or HA-6KR from cell lysates expressing both HA and Myc tagged CK2β or 6KR. Similar to wild-type CK2β, only when both Myc-6KR and HA-6KR were present could binding be detected (Fig. 4B). These results clearly indicate that 6KR is capable of forming CK2β dimers in cells, suggesting that it should be incorporated into tetrameric complexes.

To assess the ability of 6KR to bind the catalytic CK2 subunits several experiments were conducted. COS7 cells transfected with either HA-CK2β or HA-6KR in the presence or absence of GFP-CK2α were immunoprecipitated with anti-HA and immunoblotted with GFP and CK2α antibodies (Fig. 4C, upper and middle panels). Distinct GFP-CK2α bands could only be detected when both GFP-CK2α and HA-CK2β or HA-6KR were present (Fig. 4C). Cell lysates were immunoprecipitated with anti-CK2α antibodies, isolating both endogenous CK2α and GFP-CK2α followed by immunoblotting with anti-HA (Fig. 4C, lower panel). Again, both HA-CK2β and HA-6KR were capable of forming complexes with CK2α with similar efficiency. Similarly, the ability of HA-CK2β or HA-6KR to form complexes with endogenous CK2α or CK2α′ was examined by...
immunoprecipitation. HA-CK2β and HA-6KR were able to form complexes with both endogenous CK2α and CK2α’ (Fig. 5A). Collectively these data suggest that 6KR is able to form functional CK2 tetramers, because there was a clear association between both exogenous and endogenous CK2α (or endogenous CK2α’) and HA-6KR and because autophosphorylated forms of 6KR were also detected.

Immunokinase assays were used to examine the ability of 6KR to form active CK2 complexes in cells. Lysates from COS7 cells transfected with HA-CK2β or HA-6KR, were immunoprecipitated with anti-HA antibodies and immunocomplexes were utilized in kinase assays with α-casein as a substrate. No phosphorylated α-casein was detected in anti-HA immunoprecipitations from mock transfected (control) cells (Fig. 5B). However, immunoprecipitates from HA-CK2β or HA-6KR transfected cells, efficiently phosphorylated α-casein, indicating formation of active CK2 complexes. An anti-CK2β immunoblot shows that HA-CK2β or HA-6KR protein was present in each reaction and that a small amount of endogenous CK2β protein, presumably pulled down in heterogeneous CK2 complexes, was also present (lower panel). These results indicate not only that 6KR is able to form active CK2 complexes in cells, but also that 6KR does not inhibit CK2 kinase activity.

**Effect of 6KR on Cell Cycle**—Since some previous studies suggest that high levels of CK2β protein affects cell proliferation, and because we have successfully created a degradation resistant form of CK2β, we began to investigate the effect of 6KR on the cell cycle. To facilitate these studies, HeLa cells were employed. As in COS7 cells, the steady-state level of 6KR and phosphorylated 6KR protein was elevated compared with CK2β (Fig. 6A). As a first step toward investigating the effect of 6KR on the cell cycle we hypothesized that a cell cycle arrest may be occurring. HeLa cells were transfected with HA-CK2β or HA-6KR and EGFP (transfection marker) and collected 1, 2, 3, and 5 days after transfection. After propidium iodide stain-
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Effect of 6KR on Cell Proliferation—To evaluate whether HA-6KR affects cell proliferation, HeLa cells were transfected with HA-CK2β/H9252 or HA-6KR along with a puromycin resistance gene followed by selection with puromycin until colonies formed (7 days). Colonies were stained with methylene blue to assess the proliferative effects. Cells transfected with the puromycin resistance gene alone survived puromycin selection and formed colonies, while the cells lacking the puromycin resistance gene did not survive selection (Fig. 6C). Cells were also transfected with p27kip1, a cell cycle inhibitor whose overexpression blocks proliferation through G₁ arrest, but does not affect viability (38). Cells remaining on this plate represent those that were transfected and thus were resistant to puromycin treatment but were unable to proliferate due to p27kip1. These cells represent the experimental background. Transfection with HA-CK2β had no effect on cell proliferation while HA-6KR efficiently inhibited proliferation (Fig. 6C). Quantitation revealed that HA-6KR reduced proliferation to ~25% of control or HA-CK2β cells in comparison to the p27kip1-expressing cells (Fig. 6D). Transfection with CK2α-HA or HA-CK2α' alone had no effect on cell proliferation. These results suggest that stabilization of CK2β leads to a significant decrease in cell proliferation that is not mediated by the catalytic subunits and may have implications in cellular processes such as the cell cycle, apoptosis, or in cancer.

Investigation of the Cell Proliferation Defect—Inhibition of cell proliferation upon stabilization of CK2β raises numerous questions relating to whether the defect is related to the CK2 dependent or independent functions of CK2β. For example 6KR could be exerting its effect through interactions with other interactors such as A-Raf, Chk1, c-mos, or other proteins. Alternatively, the decrease in cell proliferation could be due to the effect that stabilization has on the CK2 complex.

To address both of these issues we conducted [35S]methionine cell labeling experiments on HeLa cells transfected with HA-CK2β, HA-6KR, CK2α-HA and Myc-CK2β, CK2α-HA and Myc-6KR or left untransfected (control). After an anti-HA immunoprecipitation, the bound, labeled proteins were separa-
rated and visualized by autoradiography. As expected, there was a significant increase in the steady-state expression level of HA-6KR both in the presence and absence of CK2α (Fig. 7). Interestingly, there was a significant increase in the amount of phosphorylated HA-6KR but not HA-CK2β in the presence of CK2α confirming that HA-6KR readily forms active CK2 complexes consistent with our previous data. It is also interesting to note that there is no dramatic increase in the amount of CK2α in the presence of HA-6KR compared with HA-CK2β suggesting that there has not been an increase in overall CK2 complex expression due to the presence of HA-6KR. In addition we did not observe the presence of any obvious interaction partners by which 6KR might be mediating its effect on cell proliferation. Overall, these observations suggest that the effect of 6KR does not arise from stabilization of the CK2 complex or from major changes in interactions with other proteins but does not exclude the possibility that 6KR is influencing the actions of downstream targets or CK2 selectivity.

**DISCUSSION**

Previous studies indicated that CK2β was ubiquitinated in cells and degraded by the proteasome (27). Given this information, we hypothesized that removal of one or more lysine residues would stabilize CK2β protein in cells by preventing the covalent attachment of ubiquitin. Examination of the crystal structure of CK2β revealed that of the nine lysine residues in CK2β, six were surface accessible, making them ideal sites for ubiquitin attachment (39, 40).

Although mutation of each surface-exposed lysine on its own showed no significant alterations in steady-state levels compared with wild-type CK2β, mutation of five or more lysines (with the exception of lysine 134 and 147) led to elevated steady-state expression levels and loss of sensitivity to proteasomal degradation. Because ubiquitin attachment is not highly site-specific, the fact that mutation of several lysine residues was required to prevent CK2β protein degradation by the proteasome is not surprising. The greatest enhancement in steady-state protein levels in cells occurred with the 6KR mutant, in accordance with the hypothesis that modification of the surface accessible ubiquitin attachment sites would prevent proteasomal degradation.

Previous studies demonstrated that only a portion of newly synthesized CK2β incorporates into CK2 tetramers and is stabilized, whereas free CK2β is rapidly degraded, establishing steady-state levels of CK2β within the cell (32). Given these findings it is postulated that overexpression of CK2β alone would result in the majority of new CK2β being rapidly degraded, while endogenous CK2β would remain stably associated with the CK2 catalytic subunits. In this context, the relative degradation rate of CK2β and 6KR was particularly interesting. Three hours after inhibition of protein synthesis, CK2β protein levels had significantly decreased while the 6KR protein level had not. Taken together, these results and the observation that 6KR was no longer sensitive to proteasome inhibition suggest that mutation of surface exposed lysines on CK2β (6KR) increases its half-life to at least 3 h and ablates its susceptibility to proteasomal degradation, resulting in a stabilized protein.

While 6KR results in stabilization of CK2β, other characteristics of CK2β are not altered. The 6KR protein forms dimers with wild-type and 6KR molecules indicating that the zing-finger region is not disrupted by mutating lysine 139 to arginine within this region. Furthermore, 6KR could associate with GFP-CK2α as well as endogenous CK2α or CK2α’, demonstrating that neither lysine 208 nor 212, in the positive regulatory region of CK2β, are key residues mediating binding with the catalytic subunits. In addition, slightly slower migrating bands, representing autophosphorylated forms of CK2β were detected in lanes where CK2 complex formation was observed. Because auto-phosphorylation of CK2β most likely occurs within a supramolecular complex, this implies that CK2 tetramers formed are likely functional. Phosphorylation of α-casein in 6KR immunoprecipitates provides further evidence that active, stable CK2 tetramers containing 6KR had formed in cells.

Creation of a stabilized form of CK2β that appears to function normally as a regulatory subunit of CK2 is intriguing given the involvement of CK2β in cancer and its CK2-dependent and CK2-independent roles. Although this achievement is significant on its own, we wondered what the physiological effects of CK2β stabilization might be. The traditional tetrameric view of CK2, that CK2β functions within the tetramer to modulate CK2 activity, has underscored the importance of understanding the function and regulation of CK2β. Identification of catalytic subunit independent interactors of CK2β (A-Raf, c-Mos, and Chk1) as well as differences in subcellular localization of the subunits are just two of the lines of evidence supporting CK2 independent roles for CK2β. This evidence suggests a need for investigating how CK2β protein levels are regulated and the cellular consequences of disrupting this regulation.

In past studies, the effect of CK2β overexpression on fundamental cellular processes such as cell proliferation yielded conflicting results. One study suggested that overexpression of
CK2\(\beta\) led to inhibition of cell proliferation in Chinese hamster ovary (CHO) cells and that this defect was due to disruption of the G\(_1\) phase of the cell cycle (13). By comparison, subsequent studies using human osteosarcoma (U2OS) cells stably expressing CK2\(\beta\) and mouse 3T3-L1 cells, found that CK2\(\beta\) overexpression had no effect on cell proliferation (14, 15). In this report we show that overexpression of wild-type CK2\(\beta\) in HeLa cells does not affect cell proliferation. However, overexpression of 6KR, which is degradation resistant, leads to a significant decrease in cell proliferation. Because CK2 holoenzyme levels do not significantly change when 6KR is present it is suspected that the anti-proliferative effects are due to 6KR rather than the CK2 holoenzyme. This is supported by the proliferation assays where neither CK2\(\alpha\)-HA nor HA-CK2\(\alpha\)' affect proliferation on their own.

Elevation of CK2\(\beta\) protein levels alone however, may not influence cell proliferation rate, but rather, it may be a downstream consequence of CK2\(\beta\) protein level elevation. In our cell labeling experiments we did not observe any obvious interaction partners of 6KR that could explain the inhibition of cell proliferation. Similarly in large-scale immunoprecipitation experiments we did not observe any major bands that increased or decreased in the presence or absence of 6KR (data not shown) suggesting that rather than affecting one or two protein interactions, the effect may be more widespread in its consequences.

The involvement of CK2 at almost all stages of the cell cycle and in particular the G\(_0\)/G\(_1\), G\(_1\)/S, and the G\(_2\)/M transitions raises the prospect that expression of 6KR could alter the rate of cell cycle progression (3, 6, 7, 44, 45). In light of these complex roles of CK2 in the control of proliferation, it is perhaps not surprising that we did not observe any specific changes in cell cycle profiles of cells transfected with 6KR as compared with cells transfected with CK2\(\beta\). In a similar respect, it is also intriguing that CK2 has both apoptotic and anti-apoptotic roles, both of which may be affected by stabilization of CK2\(\beta\) (46). Examination of FACS profiles (as illustrated in Fig. 6B) does not provide any indication of increased apoptosis in cells expressing 6KR as illustrated by the absence of cells exhibiting a sub G\(_0\)/G\(_1\) DNA content suggesting that apoptosis is not the sole event responsible for the attenuated proliferation observed with 6KR. Overall, given that CK2 has been implicated in a broad series of cellular events linked to proliferation, it is very likely that a number of distinct pathways and/or CK2 substrates contribute to the effects that we observe. Accordingly, elucidation of the precise mechanistic basis for the role of 6KR will require detailed systematic studies.

Based on the recent emergence of CK2\(\beta\)-independent roles for CK2\(\beta\), stabilization of the CK2\(\beta\) subunit also has the potential to significantly affect a number of other signal transduction pathways through binding to A-Raf, c-Mos, or Chk1 (47–51). At this point it is not known whether 6KR will retain its ability to associate with each of these protein kinases, however, because 6KR retained binding to the catalytic subunits of CK2 it is anticipated that 6KR will also form complexes with A-Raf, c-Mos, and Chk1. Assuming that each kinase is able to bind 6KR, it is expected that expression of 6KR will elevate A-Raf activity toward MEK. Similarly 6KR expression is also expected to elevate Chk1 activity. Indeed Chk1 lacking its C-terminal region, which is hypothesized to have an autoinhibitory role, is 20-fold more active, and it is currently thought that binding of CK2\(\beta\) to full-length Chk1 alleviates this autoinhibition (50). Furthermore, studies by Chen and colleagues proposed that CK2\(\beta\) binds and inhibits c-Mos during its initial synthesis (48), and this inhibition is alleviated once c-Mos overcomes a certain threshold that is set by the amount of free CK2\(\beta\) available (48), thus allowing for activation of the MAPK pathway. Accordingly, this would make it possible that expression of stabilized CK2\(\beta\) would disrupt c-Mos signaling by significantly elevating the threshold level of free CK2\(\beta\) that must be overcome by c-Mos to activate the MAPK pathway.

In closing, our data demonstrate that a stabilized form of CK2\(\beta\) can be generated by mutating its six surface-accessible lysines to arginine. Evaluation of this mutant demonstrates that, unlike the catalytic subunits of CK2, which promote proliferation, expression of this stabilized form of CK2\(\beta\) can be used to inhibit proliferation. Given the evidence that CK2 is overexpressed in tumors and that the catalytic CK2 subunits exhibit oncogenic activity and promote transformation, our observation that a stabilized form of CK2\(\beta\) is more effective than wild-type CK2\(\beta\) in inhibiting proliferation raises interesting prospects for targeting CK2 for therapeutic intervention.

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REFERENCES

1. Meggio, F., and Pinna, L. A. (2003) *FASEB J.* 17, 349–368
2. Kikkawa, U., Mann, S. K., Firtel, R. A., and Hunter, T. (1992) *Mol. Cell. Biol.* 12, 5711–5723
3. Glover, C. V. (1998) *Prog. Nucleic Acids Res. Mol. Biol.* 59, 95–133
4. Lorenz, P., Pepperkok, R., Ansorge, W., and Pyerin, W. (1993) *J. Biol. Chem.* 268, 2733–2739
5. Pepperkok, R., Lorenz, P., Jakobi, R., Ansorge, W., and Pyerin, W. (1991) *Exp. Cell Res.* 197, 245–253
6. Pepperkok, R., Lorenz, P., Ansorge, W., and Pyerin, W. (1994) *J. Biol. Chem.* 269, 6986–6991
7. Hanna, D. E., Rethinaswamy, A., and Glover, C. V. (1995) *J. Biol. Chem.* 270, 25905–25914
8. Daya-Makin, M., Sanghera, J. S., Mogentale, T., Lipp, M., Parchomchuk, J., Hogg, J., and Pelech, S. (1994) *Cancer Res.* 54, 2262–2268
9. Stalter, G., Siemer, S., Becht, E., Ziegler, M., Remberger, K., and Issinger, O. G. (1994) *Biochem. Biophys. Res. Commun.* 202, 141–147
10. Yenice, S., Davis, A. T., Goueli, S. A., Akdas, A., Limas, C., and Ahmed, K. (1994) *Prostate* 24, 11–16
11. Faust, R. A., Gapany, M., Tristani, P., Davis, A., Adams, G. L., and Ahmed, K. (1996) *Cancer Lett.* 101, 31–35
12. Landsman-Bollag, E., Romieu-Mouruez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D., and Seldin, D. C. (2001) *Oncogene* 20, 3247–3257
13. Li, D., Dobrowolska, G., Aicher, L. D., Chen, M., Wright, J. H., Drueckes, P., Dunphy, E. L., Munar, E. S., and Krebs, E. G. (1999) *J. Biol. Chem.* 274, 32988–32996
14. Lebrin, F., Chambaz, E. M., and Bianchini, L. (2001) *Oncogene* 20, 2010–2022
15. Vilk, G., Derksen, D. R., and Litchfield, D. W. (2001) *J. Cell Biochem.* 84, 84–99
16. Pinna, L. A. (2002) *J. Cell Sci.* 115, 3873–3878
17. Litchfield, D. W., Lozeman, F. J., Piening, C., Sommercorn, J., Takio, K., Walsh, K. A., and Krebs, E. G. (1990) *J. Biol. Chem.* 265, 7638–7644
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18. Lozeman, F. J., Litchfield, D. W., Piening, C., Takio, K., Walsh, K. A., and Krebs, E. G. (1990) Biochemistry 29, 8436–8447
19. Litchfield, D. W., Bosc, D. G., Canton, D. A., Saulnier, R. B., Valk, G., and Zhang, C. (2001) Mol. Cell. Biochem. 227, 21–29
20. Allende, I. E., and Allende, C. C. (1995) FASEB J. 9, 313–323
21. Giertz, R. D., Graham, K. C., and Litchfield, D. W. (1995) J. Biol. Chem. 270, 13017–13021
22. Boldyreff, B., Mietens, U., and Issinger, O. G. (1996) FEBS Lett. 379, 153–156
23. Marin, O., Meggio, F., Sarno, S., and Pinna, L. A. (1997) Biochemistry 36, 7192–7198
24. Graham, K. C., and Litchfield, D. W. (2000) J. Biol. Chem. 275, 5003–5010
25. Chantalat, L., Leroy, D., Filhol, O., Nueda, A., Benitez, M. J., Chambaz, E. M., Cochet, C., and Dideberg, O. (1999) EMBO J. 18, 2930–2940
26. Canton, D. A., Zhang, C., and Litchfield, D. W. (2001) Biochem. J. 358, 87–94
27. Zhang, C., Valk, G., Canton, D. A., and Litchfield, D. W. (2002) Oncogene 21, 3754–3764
28. Litchfield, D. W., Lozeman, F. J., Cicirelli, M. F., Harrylock, M., Ericsson, L. H., Piening, C. J., and Krebs, E. G. (1991) J. Biol. Chem. 266, 20380–20389
29. Litchfield, D. W., Bosc, D. G., and Slominski, E. (1995) Biochim. Biophys. Acta 1269, 69–78
30. Meggio, F., Boldyreff, B., Marin, O., Issinger, O. G., and Pinna, L. A. (1995) Eur. J. Biochem. 230, 1025–1031
31. Boldyreff, B., James, P., Staudenmann, W., and Issinger, O. G. (1993) Eur. J. Biochem. 218, 515–521
32. Luscher, B., and Litchfield, D. W. (1994) Eur. J. Biochem. 220, 521–526
33. Penner, C. G., Wang, Z., and Litchfield, D. W. (1997) J. Cell Biol. 64, 525–537
34. Bosc, D. G., Graham, K. C., Saulnier, R. B., Zhang, C., Prober, D., Giertz, R. D., and Litchfield, D. W. (2000) J. Biol. Chem. 275, 14295–14306
35. Litchfield, D. W., Luscher, B., Lozeman, F. J., Eisenman, R. N., and Krebs, E. G. (1992) J. Biol. Chem. 267, 13943–13951
36. Laemmli, U. K. (1970) Nature 227, 680–685
37. Pickart, C. M. (2001) Mol. Cell 8, 499–504
38. Sherr, C. J., and Roberts, J. M. (1999) Genes Dev. 13, 1501–1512
39. Niefind, K., Guerra, B., Ermakowa, I., and Issinger, O. G. (2001) EMBO J. 20, 5320–5331
40. Berman, H. M., Westbrook, J., Feng, Z., Gililand, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) Nucleic Acids Res. 28, 235–242
41. Deleted in proof
42. Deleted in proof
43. Deleted in proof
44. Bosc, D. G., Luscher, B., and Litchfield, D. W. (1999) Mol. Cell. Biochem. 191, 213–222
45. Issinger, O. G. (1993) Pharmacol. Ther. 59, 1–30
46. Litchfield, D. W. (2003) Biochem. J. 369, 1–15
47. Boldyreff, B., and Issinger, O. G. (1997) FEBS Lett. 403, 197–199
48. Chen, M., Li, D., Krebs, E. G., and Cooper, J. A. (1997) Mol. Cell. Biol. 17, 1904–1912
49. Hagemann, C., Kalmes, A., Wixler, V., Wixler, L., Schuster, T., and Rapp, U. R. (1997) FEBS Lett. 403, 200–202
50. Guerra, B., Issinger, O. G., and Wang, J. Y. (2003) Oncogene 22, 4933–4942
51. Lieberman, S. L., and Ruderman, J. V. (2004) Dev. Biol. 268, 271–279