Assembly of protein kinase CK2: investigation of complex formation between catalytic and regulatory subunits using a zinc-finger-deficient mutant of CK2β

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Protein kinase CK2 is a tetrameric enzyme comprised of two regulatory subunits (CK2β) and two catalytic subunits (CK2α and/or CK2α'). The crystal structure of dimeric CK2β demonstrated that a zinc finger mediates CK2β dimerization, therefore we constructed a mutant in which cysteine residues 109 and 114 were mutated to serine. Our objectives were to examine the effects of disrupting the zinc finger of the regulatory CK2β subunit on CK2 tetramer assembly. Examination of this zinc-finger-deficient mutant of CK2β using a yeast two-hybrid assay demonstrates that the mutant fails to form CK2β homodimers. In order to extend these studies, we co-transfected COS-7 cells with epitope-tagged constructs and performed co-immunoprecipitation assays. The results from these studies demonstrate that the mutant fails to form CK2β homodimers and fails to interact with catalytic CK2 subunits. Furthermore, we demonstrate that the mutant CK2β is not appreciably phosphorylated in cells. Using *in vitro* binding assays, we demonstrated that the mutant CK2β protein fails to interact with glutathione S-transferase–CK2α'. Finally, we demonstrate that the mutant is translated at an equivalent rate to wild-type CK2β, but is degraded much more rapidly. Overall, our results are consistent with the model that β–β dimerization precedes incorporation of catalytic subunits into tetrameric CK2 complexes, and that β–β dimerization is a prerequisite for the stable incorporation of catalytic subunits into CK2 complexes.

Key words: protein phosphorylation, signal transduction, mutagenesis.

INTRODUCTION

Protein kinase CK2 (an acronym derived from the misnomer 'casein kinase II') is a ubiquitous and highly conserved serine/threonine protein kinase present in all eukaryotic cells [1–6]. Although its precise biological functions remain poorly understood, CK2 is essential for viability in eukaryotic organisms [7–9]. There is mounting evidence to suggest that CK2 plays an important role in control of cell proliferation and transformation [3–5,10]. A number of studies have shown alteration in the expression of CK2 in a variety of tumour or leukaemic cells [11–14]. For example, the targeted overexpression of CK2α in the T-cells of transgenic mice results in lymphocyte transformation. Furthermore, there is evidence for collaboration between the dysregulated expression of CK2α and the c-Myc and Tal-1 oncogenes in lymphoma development [15,16]. Collectively, these studies point to a role for CK2 as a component of the kinase networks that regulate growth and division of cells.

CK2 has been reported to phosphorylate and regulate the activity of numerous proteins involved in cellular processes such as transcription, translation, morphogenesis and cell cycle regulation [1–6,10]. However, its specific modes of regulation in intact cells remain poorly understood. The majority of CK2 in cells exists as a tetrameric protein comprised of two regulatory subunits (CK2β) and two catalytic subunits (CK2α and/or CK2α'). Despite being the products of separate genes, the two catalytic subunits exhibit greater than 90% sequence identity in the N-terminal 330 amino acids [10]. Conversely, the C-terminal domains of the two isoenzymes are completely unrelated ([17], but see [17a], [18,19]). The regulatory subunit, CK2β, does not share extensive similarity with any known protein. However, it exhibits remarkable conservation between species as shown by the fact that the deduced amino acid sequences of human and chicken CK2β are identical. Furthermore, the deduced sequences of *Xenopus laevis* CK2β and *Danio rerio* (zebrafish) CK2β differ from those of human and chicken by only one and two amino acids, respectively [4]. Such a high degree of evolutionary conservation suggests that CK2β plays important roles in cellular functions. The regulatory subunit alone has no known catalytic activity, but it does modulate the ability of CK2α to interact with and phosphorylate substrate proteins [20–23]. Furthermore, CK2β appears to mediate the effects of polyamines that may play a role in regulating CK2 activity in cells [24,25]. Collectively, these studies suggest that the regulatory subunit is a crucial mediator of the cellular functions of CK2.

To fully define the functions and regulation of CK2 *in vivo*, an understanding of the process of CK2 tetramer assembly is critical. To this end, there is mounting evidence to suggest that the formation of complexes between CK2β subunits precedes the incorporation of catalytic subunits into tetrameric complexes. We have previously demonstrated that the β-subunit is synthesized in excess of α and interacts slowly with α [26]. However, the majority of newly synthesized α-subunits is rapidly incorporated into tetrameric complexes with β-subunits. This finding suggests that β–β dimerization precedes tetramer formation of CK2. In studies using the yeast two-hybrid system, we

Abbreviations used: CK2, protein kinase CK2 or casein kinase II; CMV, cytomegalovirus; GST, glutathione S-transferase; k.d., kinase dead; PBST, 0.1% Tween 20 in PBS.

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and others have shown that CK2β subunits can interact with both α and β, while CK2α subunits can only interact with β [27–29]. Furthermore, we have demonstrated that complexes between two CK2β subunits can form in mammalian cells in the absence of catalytic subunit [30]. Significantly, this study suggested that each CK2β subunit interacts with both catalytic subunits in tetrameric CK2. Recently, a crystal structure of dimeric CK2β was reported [31]. This structure suggests that β–β dimerization is mediated through a zinc finger that is composed of four conserved cysteine residues: Cys^{109}, Cys^{114}, Cys^{137} and Cys^{140}. Importantly, the fact that CK2β subunits can associate in the absence of CK2α suggests that the crystal structure of dimeric CK2β does indeed represent a physiologically relevant structure [31].

Following publication of the structure of dimeric CK2β, Meggio et al. [32] demonstrated that recombinant CK2β treated with sulphydryl-modifying reagents fails to form homodimers, but forms $\alpha\beta$ dimers, and $\alpha_2\beta/\alpha\beta_2$ trimers, *in vitro*. Since CK2β exerts both positive and negative regulatory effects on CK2 activity [20,30,32a] and because one CK2β subunit appears to interact with both catalytic subunits within the CK2 tetramer [30], we postulated that $\alpha\beta$ dimers, which are unable to form tetrameric complexes, may exhibit dysregulated CK2 activity. To test this prediction, we constructed a Myc-tagged CK2β mutant in which two of the conserved zinc-finger residues, Cys^{109} and Cys^{114}, were mutated to serine. Our objectives were to study the effects of disrupting the zinc finger of CK2β on the formation of multi-subunit CK2 complexes, and to evaluate the usefulness of such a mutant as a dominant unregulated mutant of CK2. Results from this study indicate that the zinc finger mutant is neither able to form homodimers nor able to interact with the catalytic subunits of CK2 either *in vitro* or in cells. Furthermore, we demonstrate that the mutant is not appreciably phosphorylated in cells and is rapidly degraded. Importantly, these results are in good accordance with a model whereby complexes between CK2β subunits precede the formation of tetrameric CK2 complexes, and whereby formation of CK2β homodimers is required for the incorporation of catalytic subunits into tetrameric CK2 complexes.

EXPERIMENTAL

Antibodies

Purified 12CA5 monoclonal antibodies directed at the HA epitope [33] (the YPYDVPDY epitope of influenza virus haemagglutinin) were purchased from Berkeley Antibody (Richmond, CA, U.S.A.). The hybridoma producing 9E10 monoclonal antibody against the Myc epitope [34] (the MASMEQKLISEEDLNN epitope of the c-Myc protein) was obtained from the A.T.C.C. (Manassas, VA, U.S.A.). Cells were used to produce ascites in mice and antibodies were partially purified by ammonium sulphate precipitation.

Plasmid constructs

Full-length HA-tagged CK2α', HA-CK2β and Myc-CK2β containing N-terminal HA or Myc tags, respectively, were expressed using pRc/cytomegalovirus (CMV) as described previously [23,30]. A kinase-inactive [kinase dead (k.d.)] mutant of HA-α' (HA-α'k.d.) was constructed by mutating the codon for lysine to methionine at amino acid position 69 as described previously [18,35]. A zinc-finger-disrupted CK2β construct was generated using site-directed mutagenesis to mutate cysteine residues 109 and 114 to serine (designated Myc$\beta^{C109,114S}$). Constructs encoding full-length CK2β and CK2$\beta^{C109,114S}$ fused to the transcriptional activation domain of Gal4 were generated by subcloning Mycβ and Myc$\beta^{C109,114S}$ out of pRc/CMV using *Xho*I and ligating the 675 bp fragment into the *Xho*I site of pACT2. Constructs were verified by sequencing.

Transformation and maintenance of yeast

Yeast strains were grown and manipulated according to standard methods [36]. For two-hybrid assays, the yeast strain CTY::171 (*MATa ade2 gal4 gal80 his3-Δ200 leu2-3 113 trpl-Δ901 ura3-52 URA3::GAL1-lacZ*) was transformed with equal quantities of pGBT9 and pACT2 plasmids using the method described by Gietz et al. [37]. Transformants were selected for on synthetic complete medium minus Trp and Leu plates at 30 °C, and grown for 3–4 days.

Measurement of β-galactosidase activity

Assays for β-galactosidase were performed as described previously [27]. Briefly, multiple colonies were scraped off each plate and transferred to 1 ml of distilled water. After thorough mixing, D_{600} measurements of each suspension were taken so that β-galactosidase activities could be normalized to the density of yeast cells. Equivalent units of yeast were transferred to microfuge tubes and pelleted by brief centrifugation. The supernatant was removed and the cells were resuspended in 500 μl of Z buffer (100 mM $NaPO_4$, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$ and 38 mM β-mercaptoethanol). A 50 μl aliquot of 0.1 % SDS was added and the cells were vortexed vigorously for 15 s. Chloroform (50 μl) was then added and the cells were vortexed for an additional 15 s. Assays for β-galactosidase were immediately initiated by the addition of 100 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside and incubations were conducted at 37 °C. Reactions were terminated by the addition of 500 μl of 1 M Na_2CO_3. Reaction mixtures were centrifuged for 1 min and the A_{420} was measured. β-Galactosidase activities in Miller units were calculated according to the formula as follows:

$$\text{Activity} = A_{420}/(V \cdot t \cdot D_{600}) \times 1000$$

Where V is the volume of the cuvette (1 ml), and t is the time in min. Results were expressed relative to the background pGBT9/pACT2 activity, and values are the mean of three independent experiments.

Transfection of COS-7 cells and cycloheximide treatment

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (Gibco BRL) at 37 °C in an atmosphere of 5 % CO_2. Cells were transfected using the calcium phosphate precipitation method as described previously [23] using 125 μg of DNA per 15-cm diameter plate. Co-transfection experiments used equivalent amounts of DNA for each plasmid unless otherwise indicated. At 16–18 h after transfection, cells were washed thoroughly with PBS and re-fed with fresh medium. The cells were harvested 36–48 h later. For examination of protein turnover, COS-7 cells transfected with either Mycβ or Myc$\beta^{C109,114S}$ were treated with 20 μl of cycloheximide (50 mg/ml in 95 % ethanol) per 15-cm diameter plate. Cells were then harvested at various time intervals and 30 μg of protein was analysed by SDS/PAGE and immunoblotting.

Cell labelling and lambda phosphatase treatment

For cell labelling experiments, COS-7 cells grown in 60-mm diameter plates were transfected with HA- and Myc-tagged

constructs as indicated. At 36–48 h following transfection, cells were labelled with [^{35}S]methionine for 4 h at 37 °C. In the case of Mycβ-transfected cells, labelling was with 0.2 mCi [^{35}S]-methionine, while cells transfected with Myc$\beta^{C109,114S}$ were labelled with 0.5 mCi of [^{35}S]methionine. Anti-Myc immunoprecipitations were performed on lysates derived from these cells and the proteins were separated by SDS/PAGE as described below. The radiolabelled proteins were revealed using a PhosphorImager (Molecular Dynamics).

For phosphatase treatment, 400 units of Lambda protein phosphatase (Cell Signaling Technology, Beverly, MA, U.S.A.) was added to 50 μg of the total cell lysate in the presence of 2 mM $MnCl_2$. Following incubation at 30 °C for 30 min, proteins were separated by SDS/PAGE and transferred to PVDF membranes for immunoblotting as indicated.

Immunoprecipitations

For immunoprecipitations, COS-7 cells were lysed on ice in 0.5 ml of Nonidet P40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Nonidet P40, 1 % aprotinin and 0.1 mM PMSF) and scraped off the plates. The lysates were sonicated on ice with three 10 s bursts, and then centrifuged at 16000 ***g*** in a Beckman TL100.2 rotor for 15 min at 4 °C. The cleared lysates were subjected to immunoprecipitation. Antibodies used were either 12CA5 or 9E10 monoclonal antibodies covalently linked to Protein A–Sepharose using rabbit anti-mouse antibody as a cross-linker. Following incubation for 1 h at 4 °C, the lysates were centrifuged briefly and the Protein A–Sepharose beads were washed four times with lysis buffer. Following the last wash, proteins bound to the beads were eluted by the addition of 2 × Laemmli buffer [38].

Immunoblot analysis

Samples were separated by SDS/PAGE (12 % gel) using the method of Laemmli [38]. Proteins were transferred to PVDF membranes for 1 h at 100 V in blotting buffer (25 mM Tris, 190 mM glycine and 20 % methanol) as described by Towbin et al. [39]. Anti-HA blots were performed as described previously [23] using 12CA5 monoclonal antibody (1:500 dilution) and immune complexes were detected using goat anti-mouse antibody (1:3000 dilution) conjugated to alkaline phosphatase for colour development with bromochloroindoyl phosphate and Nitro Blue Tetrazolium as substrates. Anti-Myc blots were performed using a low salt method as described below. Membranes were blocked for 1 h with 3 % BSA in PBS with 0.1 % Tween 20 (PBST). The membrane was washed three times with PBST and reacted with 9E10 monoclonal antibody (1:2000 dilution) for 16–18 h at 4 °C. Following washing with PBST, immune complexes were detected using goat anti-mouse antibody (1:20000 dilution) conjugated to horseradish peroxidase for detection by enhanced chemiluminescence using SuperSignal (Pierce).

In vitro translation assays and GST-pulldowns

^{35}S-labelled Mycβ and Myc$\beta^{C109,114S}$ were produced by *in vitro* transcription and translation using a TnT kit (Promega) with T7 polymerase according to the manufacturer's instructions. For studies on protein translation through time, 5 μl aliquots of ^{35}S-labelled Mycβ and Myc$\beta^{C109,114S}$ were removed every 15 min until the completion of the 90 min reaction. GST-fusion proteins encoding full-length CK2α' or GST alone were expressed in bacteria and purified using glutathione–agarose as described previously [40]. The purified fusion proteins were then coupled to Affi-Gel 10 (Bio-Rad) at a concentration of 4 mg/ml according to the manufacturer's instructions. Following *in vitro* transcription and translation, GST-pulldowns were performed by diluting the TnT reactions with 50 μl RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 % Nonidet P40, 0.5 % deoxycholate and 0.1 % SDS) and adding 10 μl of Affi-Gel 10 GST or GST-α' beads. Incubation was at 4 °C for 1 h with rocking. The Affi-Gel beads were collected by centrifugation, the supernatant was removed and the beads were washed four times with 100 μl RIPA buffer. Proteins bound to the Affi-Gel beads were eluted by the addition of 2 × Laemmli sample buffer and were subjected to SDS/PAGE on a 12 % gel. After drying, the radiolabelled proteins were revealed using a PhosphorImager.

RESULTS

Yeast two-hybrid assay

Our objectives were to examine the effect of a zinc-finger mutant on complex assembly between CK2 subunits. In order to characterize interactions of the zinc-finger mutant CK2$\beta^{C109,114S}$ with the various subunits of CK2, we initially utilized the yeast two-hybrid system developed by Fields and Song [41]. For these experiments, the cDNAs encoding the α, β and zinc-finger-disrupted $\beta^{C109,114S}$ subunits of CK2 were each ligated into pGBT9 and pACT2 vectors in order to express each as a fusion protein with the DNA-binding domain (pGBT9) or with the transcriptional activation domain (pACT2) of the yeast Gal4 transcriptional activator. The yeast strain CTY::171, which expresses β-galactosidase activity under the control of a Gal1 promoter that is regulated by the Gal4 transcription factor, was then co-transformed with various combinations of pGBT9 and pACT2 constructs. An indication of the interactions between fusion proteins was obtained by measuring β-galactosidase activity in extracts from the various transformants. Initial experiments were performed in order to test whether the zinc-finger mutant CK2$\beta^{C109,114S}$ was able to dimerize with wild-type CK2β. Significant interaction is observed between pGBT9-CK2β and pACT2-CK2β as expected (results not shown). However, β-galactosidase activity was not evident in yeast co-transformed with pGBT9-CK2β and pACT2-CK2$\beta^{C109,114S}$ indicating that the zinc-finger mutant is not able to dimerize with wild-type CK2β. Importantly, β-galactosidase expression is not evident in yeast co-transformed with pGTB9-CK2β and pACT2 indicating that pGBT9-CK2β alone is not capable of stimulating transcription. We then examined the interactions of CK2$\beta^{C109,114S}$ with the catalytic subunit CK2α. In this case, high β-galactosidase activity is evident in yeast co-transformed with pGBT9-CK2α and pACT2-CK2β, as well as with pGBT9-CK2α and pACT2-CK2$\beta^{C109,114S}$. Furthermore, similar results were obtained when yeast were co-transformed with pGBT9-CK2α' and pACT2-CK2β indicating that the observed interactions were not isoenzyme specific (results not shown). Importantly, no β-galactosidase expression is evident in yeast co-transformed with pGTB9-CK2α and pACT2 indicating that pGBT9-CK2α alone is incapable of activating transcription. From these results, it appears that the zinc-finger mutant CK2$\beta^{C109,114S}$ retains the ability to bind to the catalytic subunit despite the fact that it is deficient in dimerization.

Co-immunoprecipitation assays

To further characterize the zinc-finger mutant CK2$\beta^{C109,114S}$, we examined its ability to interact with CK2α and CK2β in mammalian cells using co-immunoprecipitation assays (Figure 1). To achieve this objective, we co-transfected COS-7 cells with HA-tagged constructs encoding CK2α', kinase dead CK2α'k.d.

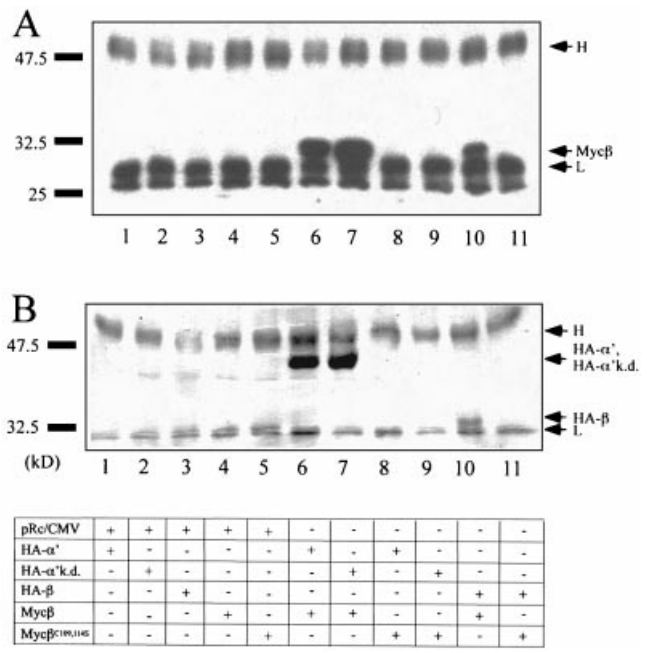

Figure 1 Co-immunoprecipitation assays to measure interactions between CK2 subunits

In order to examine the interactions of Mycβ or Myc$\beta^{C109,114S}$ with HA-α', HA-α'k.d. (with the mutation K69M) and HA-β, COS-7 cells were transfected with plasmids encoding each of the five proteins. Extracts were prepared from the transfected cells and immunoprecipitations were performed with either anti-HA 12CA5 or anti-Myc 9E10 antibodies as indicated. Immunoprecipitates were separated by SDS/PAGE and transferred to PVDF membranes for immunoblotting with either 9E10 or 12CA5 antibodies. Immune complexes were detected with enhanced chemiluminescence as described in the Experimental section. The positions of Mycβ, HA-α', HA-α'k.d. and HA-β are indicated as well as the positions of the heavy (H) and light (L) chains of IgG. (**A**) Immunoprecipitation with anti-HA, followed by immunoblot with anti-Myc. (**B**) Immunoprecipitation with anti-Myc, followed by immunoblot with anti-HA.

or CK2β together with Myc-tagged constructs encoding CK2β or the corresponding zinc-finger mutant designated CK2$\beta^{C109,114S}$. Anti-HA immunoprecipitations were performed with lysates derived from these cells and examined on Western blots with anti-Myc antibodies (Figure 1A). Alternatively, immunoprecipitations performed using anti-Myc antibodies were revealed on Western blots using anti-HA antibodies (Figure 1B). As expected, Mycβ can be isolated in complexes with HA-α' and HA-α'k.d. (Figure 1A, lanes 6 and 7). Conversely, Myc$\beta^{C109,114S}$ is not detected in the anti-HA immunoprecipitations (Figure 1A, lanes 8 and 9). This suggests that Myc$\beta^{C109,114S}$ is not able to form complexes with HA-α' or HA-α'k.d. in mammalian cells. Furthermore, Mycβ is found in complexes with HA-β as expected (Figure 1A, lane 10). However, Myc$\beta^{C109,114S}$ is not detected in anti-HA immunoprecipitations. (Figure 1A, lane 11). This suggests that the zinc-finger mutant Myc$\beta^{C109,114S}$ is not able to dimerize with HA-β *in vivo*. We substantiated these results using anti-Myc immunoprecipitations of transfected cell lysates followed by Western-blot analysis with anti-HA antibodies (Figure 1B). In agreement with the above results, HA-α' and HA-α'k.d. were both found in complexes with Mycβ (Figure 1B, lanes 6 and 7). By comparison, neither HA-α' or HA-α'k.d. were detected in complexes with Myc$\beta^{C109,114S}$ (Figure 1B, lanes 8 and 9). Similarly, while HA-β was found in complexes with Mycβ, no interaction was detected between HA-β and Myc$\beta^{C109,114S}$ (Figure 1B, lanes 10 and 11). Expression of all HA-tagged and Myc-tagged constructs was confirmed by probing anti-HA immunoprecipitations and anti-Myc immunoprecipitations with anti-HA and anti-Myc antibodies, respectively, on immunoblots (results not shown). Our results demonstrate that the zinc-finger mutant Myc$\beta^{C109,114S}$ was not able to dimerize with HA-β and was not able to form tetrameric complexes with HA-α' or HA-α'k.d. in mammalian cells.

Autophosphorylation of the regulatory CK2β subunit

Following the formation of tetrameric complexes in cells, CK2β is known to be autophosphorylated by the catalytic subunit [23]. Phosphorylated Mycβ is easily differentiated from the non-phosphorylated protein by a reduction in electrophoretic mobility during gel electrophoresis. Since we demonstrated that the zinc-finger mutant is not able to form complexes with the catalytic subunit, we postulated that Myc$\beta^{C109,114S}$ would not be phosphorylated in the presence of HA-α'. To test this hypothesis, we examined the phosphorylation state of Myc$\beta^{C109,114S}$ in mammalian cells (Figure 2). Anti-Myc immunoprecipitations were performed with lysates derived from these cells and examined by immunoblots with anti-Myc antibodies. In the absence of HA-α', Mycβ is nominally phosphorylated (Figure 2A, lane 2). This phosphorylation is likely due to complex formation with endogenous CK2α. As expected, a more significant proportion of Mycβ becomes phosphorylated when co-transfected with HA-α' (Figure 2A, lane 3). Conversely, no appreciable phosphorylation of the zinc-finger mutant Myc$\beta^{C109,114S}$ was detected in the absence or presence of HA-α' (Figure 2A, lanes 5 and 6). As a negative control, HA-α' co-transfected with pRc/CMV was included to demonstrate the presence of the light chain of IgG (Figure 2A, lanes 1 and 4).

To confirm these results and to eliminate the interfering light chain, we also performed immunoprecipitations from transfected cells labelled with [^{35}S]methionine. Anti-Myc immunoprecipitations were performed on lysates derived from these cells (Figure 2B). The specificity of these immunoprecipitations was confirmed by the absence of ^{35}S-labelled bands in anti-Myc immunoprecipitations from non-transfected and vector-control transfected cells (results not shown). As expected, a significant proportion of Mycβ becomes phosphorylated when co-transfected with HA-α' (Figure 2B, lanes 1 and 2). However, co-transfection of Myc$\beta^{C109,114S}$ with HA-α' did not result in appreciable phosphorylation of the zinc-finger mutant (Figure 2B, lanes 3 and 4). In agreement with the results obtained in Figure 2(A), these results confirm that Myc$\beta^{C109,114S}$ is not appreciably phosphorylated by the catalytic subunit.

To provide additional evidence to support the suggestion that the observed shift in electrophoretic mobility is indeed due to phosphorylation, we directly examined the effects of kinase-inactive CK2 catalytic subunits and Lambda phosphatase treatment on CK2β phosphorylation. As shown in Figure 2(C), both co-transfection of Mycβ with a kinase-inactive CK2 catalytic subunit and treatment with Lambda phosphatase resulted in a reduction in CK2β phosphorylation (Figure 2C, lanes 1, 2 and 3). Our results indicate that Myc$\beta^{C109,114S}$ is not appreciably phosphorylated by the catalytic subunit. Taken together with our results from the co-immunoprecipitation assays, these results suggest that Myc$\beta^{C109,114S}$ is not able to form tetrameric complexes in mammalian cells.

Interactions between CK2α' and Myc$\beta^{C109,114S}$ *in vitro*

Based on the results from the co-immunoprecipitation assays, we demonstrated that the zinc-finger mutant Myc$\beta^{C109,114S}$ was not capable of interacting with the catalytic subunit. However, our

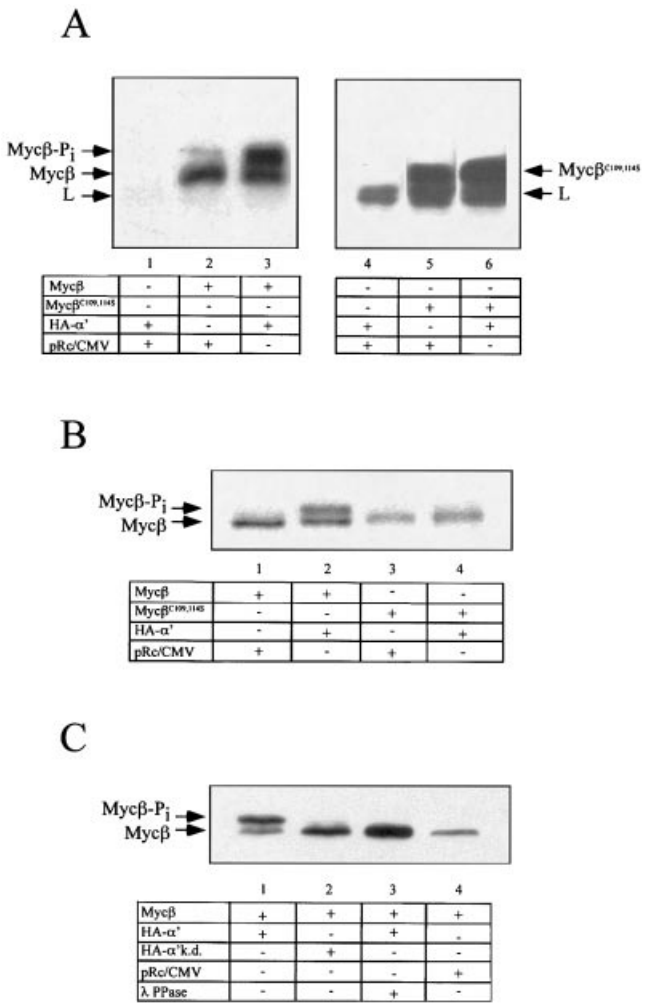

Figure 2 Examination of the phosphorylation state of CK2β and CK2$\beta^{C109,114S}$

In order to determine if Myc$\beta^{C109,114S}$ is phosphorylated in the presence of catalytic subunit, COS-7 cells were co-transfected with the plasmids indicated. (**A**) Extracts were prepared and immunoprecipitations were performed with anti-Myc antibodies. Immunoprecipitates were separated on SDS/PAGE and transferred to PVDF membranes for immunoblotting with anti-Myc antibodies, as described in the Experimental section. The positions of Mycβ, phosphorylated Mycβ (Mycβ-P$_i$), Myc$\beta^{C109,114S}$ and the light chain (L) of IgG are indicated. The light chain of IgG is only visible in lanes 4–6 because lower levels of Myc$\beta^{C109,114S}$ expression necessitated longer exposure times than those shown in lanes 1–3. (**B**) Transfected COS-7 cells were labelled with [^{35}S]methionine for 4 h and harvested as described in Experimental section. Following immunoprecipitation with anti-Myc antibodies and separation by SDS/PAGE, the gel was dried and the radiolabelled proteins were revealed using a PhosphorImager. (**C**) COS-7 cells were transfected as indicated and lysates were derived from these cells. Where indicated, Lambda phosphatase treatment was performed as described in the Experimental section. Extract protein (40μg) was separated by SDS/PAGE and transferred to PVDF membranes for immunoblotting with anti-Myc antibodies. In (**B**) and (**C**) the positions of Mycβ, phosphorylated Mycβ (Mycβ-P$_i$) and Myc$\beta^{C109,114S}$ are indicated.

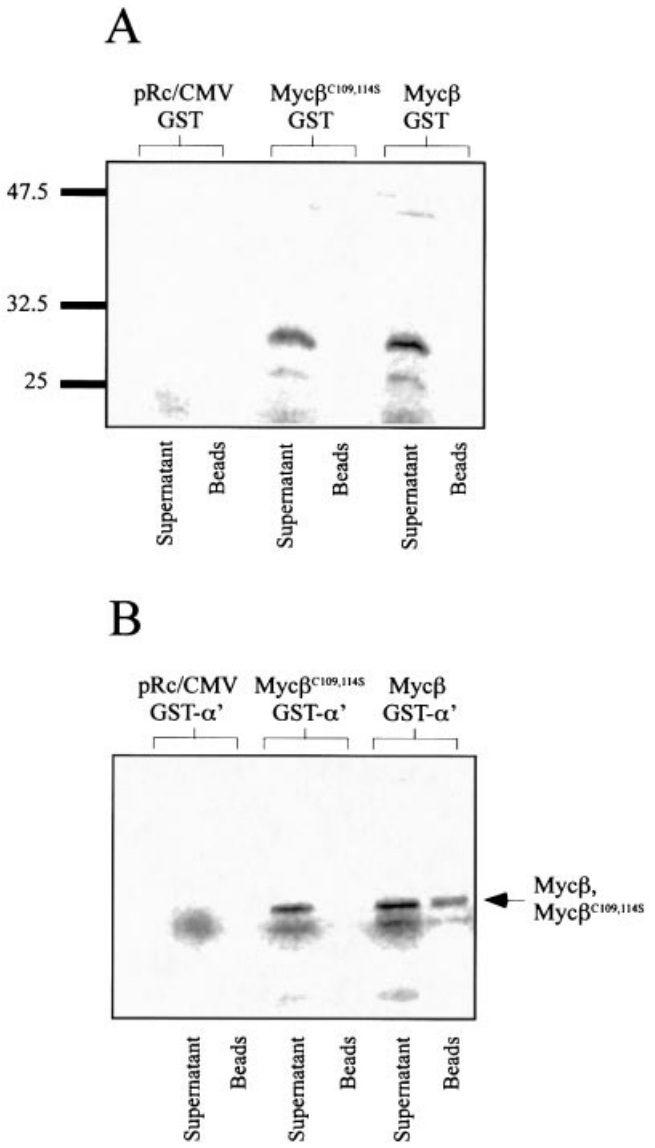

Figure 3 Interactions between CK2 subunits using *in vitro* translated proteins and GST–CK2α'

^{35}S-labelled proteins were generated by *in vitro* transcription and translation as described in the Experimental section. *In vitro* translation products were diluted with RIPA buffer and incubated with GST or GST–CK2α' (GST- α') coupled to Affi-Gel beads as described in the Experimental section. (**A**) GST pulldown: empty vector (pRc/CMV) plus GST, Myc$\beta^{C109,114S}$ plus GST, and Mycβ plus GST. (**B**) GST–CK2α' (GST–α') pulldown: pRc/CMV plus GST–CK2α', Myc$\beta^{C109,114S}$ plus GST–CK2α', and Mycβ plus GST–CK2α'. The major translation products of approx. 30 kDa that were obtained using Mycβ and Myc$\beta^{C109,114S}$ DNA as templates are indicated. Supernatant, proteins that failed to interact with GST or GST–CK2α'; Beads, proteins that bound to GST–CK2α'.

studies using the yeast two-hybrid system suggested that CK2$\beta^{C109,114S}$ was capable of binding to CK2α. In order to resolve this discrepancy, the ability of CK2α' to interact directly with Myc$\beta^{C109,114S}$ was tested *in vitro* using purified GST-fusion proteins and the products of *in vitro* transcription and translation. As a control, we demonstrated that *in vitro* translated Myc$\beta^{C109,114S}$ and Mycβ fail to interact with GST coupled to Affi-Gels as expected (Figure 3A). By using GST–CK2α' protein as an affinity matrix, it was found that CK2α' interacted with Mycβ as expected, but no interaction was observed between CK2α' and Myc$\beta^{C109,114S}$ (Figure 3B). In each case, the empty vector template pRc/CMV was included as a negative control. A lower band (i.e. just above the 25 kDa marker) that is present in both Mycβ and Myc$\beta^{C109,114S}$ could either be an alternative translation product resulting from a second start codon or a proteolytic degradation fragment. However, the precise identity of the band is unknown. The results obtained from this *in vitro* binding assay, together with the results from the co-immunoprecipitation assay, confirm

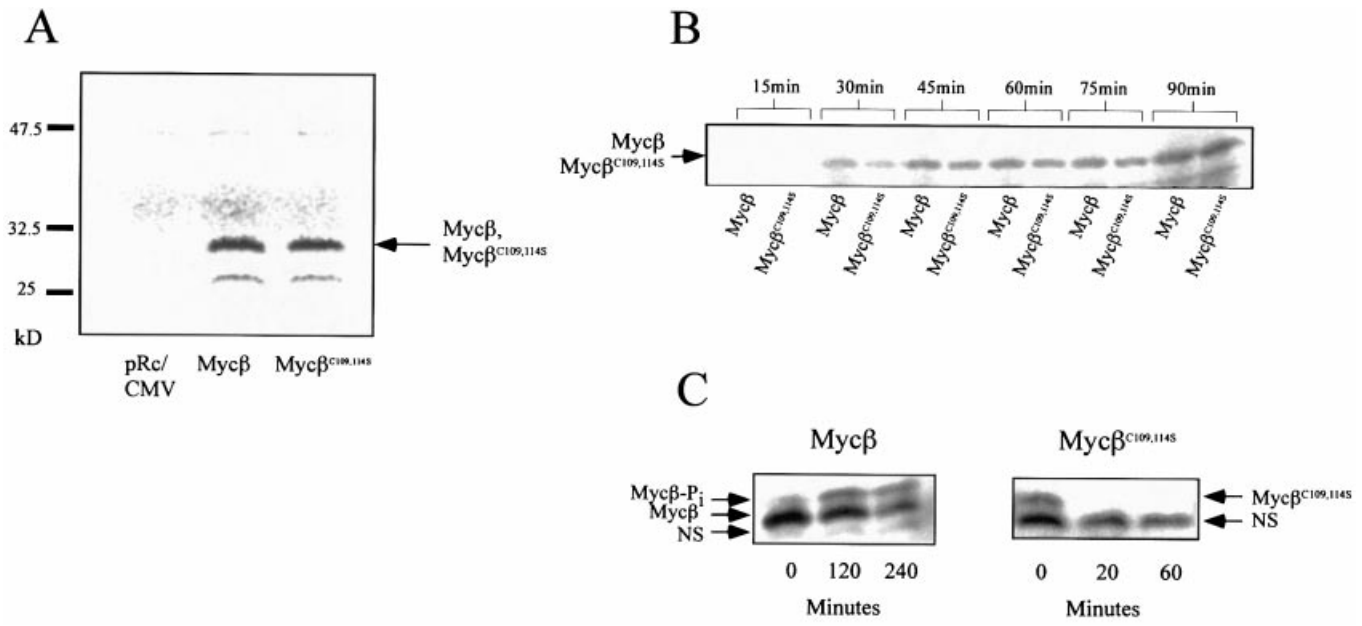

Figure 4 ***In vitro*** **translation and protein turnover of Mycβ and Myc$\beta^{C109,114S}$**

^{35}S-labelled Mycβ and Myc$\beta^{C109,114S}$ were generated using an *in vitro* transcription and translation kit as described in the Experimental section. The empty vector template pRc/CMV was included as a control. (**A**) *In vitro* translated Mycβ and Myc$\beta^{C109,114S}$ after a 90-min reaction. (**B**) Time course of *in vitro* translation of Mycβ and Myc$\beta^{C109,114S}$. The major translation products of approx. 30 kDa that were obtained using Mycβ and Myc$\beta^{C109,114S}$ DNA as templates are indicated by an arrow. For protein turnover studies, COS-7 cells transfected with Mycβ or Myc$\beta^{C109,114S}$ were treated with cycloheximide and harvested at various time points as described in the Experimental section. (**C**) Extract protein (30 μg) was separated by SDS/PAGE and transferred to PVDF membranes for probing with anti-Myc antibodies. (**C**, left panel) Mycβ proteins levels after treatment with cycloheximide for 0, 120 and 240 min. (**C**, right panel) Myc$\beta^{C109,114S}$ proteins levels after treatment with cycloheximide for 0, 20 and 60 min. Arrows indicate positions of Mycβ, phosphorylated Myc-β (Mycβ-P$_i$), Myc$\beta^{C109,114S}$ and non-specific (NS) bands. Longer exposure times in the right panel resulted in more prominent appearance of the non-specific band.

that the zinc-finger mutant Myc$\beta^{C109,114S}$ is not capable of forming stable interactions with the catalytic subunit of CK2.

In vitro translation rates

When transfected into mammalian cells, Myc$\beta^{C109,114S}$ was expressed at lower levels than Mycβ (results not shown). Furthermore, the levels of Mycβ and Myc$\beta^{C109,114S}$ expression did not result from differences in immunoprecipitation efficiency as total lysates of transfected cells had comparable differences in expression levels. Therefore, we postulated that the differences were the result of changes in translational efficiency or in protein turnover. In order to examine the first possibility, we utilized *in vitro* transcription and translation to generate radiolabelled Mycβ and Myc$\beta^{C109,114S}$ (as described in the Experimental Section). Following completion of a 90-min reaction, a major radiolabelled translation product of approximately 30 kDa was detected in lysates containing Mycβ or Myc$\beta^{C109,114S}$ DNA (Figure 4A). Importantly, no differences in the translational efficiency of the two proteins were detected. As described earlier, the lower band (i.e. slightly above 25 kDa) may be the result of an alternative start codon or proteolytic degradation. To confirm that the translation rates of the two proteins were similar throughout the 90-min reaction, we removed aliquots of the two lysates every 15 min throughout the incubation period (Figure 4B). Based on the quantification of two independent experiments (results not shown) we conclude that there is no significant difference in the translation efficiency of Mycβ and Myc$\beta^{C109,114S}$ throughout the 90-min assay. On the basis of this result, it is unlikely that a decrease in translational efficiency is responsible for the low expression of the zinc-finger mutant CK2$\beta^{C109,114S}$.

Protein turnover

In order to examine protein turnover, COS-7 cells were transfected with Mycβ or Myc$\beta^{C109,114S}$ and harvested after treatment with cycloheximide as described in the Experimental section. Cycloheximide is a protein synthesis inhibitor that functions by inhibiting translational elongation. Following cycloheximide treatment for 240 min, Mycβ was still readily detected (Figure 4C, left panel). By comparison, after cycloheximide treatment for only 20 min, Myc$\beta^{C109,114S}$ protein levels were undetectable (Figure 4C, right panel). In each case, a lower non-specific band was detected with a mass of approx. 25 kDa. This result clearly demonstrates that the zinc-finger mutant is degraded more rapidly than Mycβ and that this increase in turnover is presumably a major cause of its low expression in mammalian cells.

DISCUSSION

Recently, the crystal structure of a dimeric form of the regulatory β subunit of CK2 was reported [31]. This structure demonstrated that interaction between two CK2β subunits is mediated by a zinc finger composed of four conserved cysteine residues: Cys^{109}, Cys^{114}, Cys^{137} and Cys^{140}. In order to disrupt this zinc finger, we constructed a Myc-tagged CK2β mutant in which two of the conserved cysteine residues, Cys^{109} and Cys^{114}, were mutated to serine. Our objectives were to characterize the effects of these mutations on CK2 dimer and tetramer assembly. It has been established that CK2β plays dual roles in the regulation of CK2 catalytic activity [20,30,32a]. Studies have shown that the C-terminal domain of CK2β is responsible for stable interactions

with the catalytic subunit and for the up-regulation of catalytic activity following tetramer formation. Conversely, the N-terminal domain of CK2β has been shown to exert negative regulation on the catalytic activity of CK2. We have previously demonstrated that each CK2β-subunit interacts with both catalytic subunits in a tetramer [30]. Based on these previous results, we postulated that a dimerization-defective CK2β subunit would alleviate negative inhibition to maintain constitutively uninhibited CK2 activity. Therefore, we were interested in evaluating the usefulness of such a mutant in dominant negative and/or positive strategies. Overall, our results are consistent with the model that complexes between CK2β subunits precede incorporation of catalytic subunits into tetrameric complexes, and that formation of CK2β dimers is a prerequisite for formation of CK2 tetramers containing catalytic subunits. There is a growing body of evidence to support these conclusions. The β-subunit is synthesized in excess of the α-subunits and interacts slowly with α-subunits. Conversely, the majority of newly synthesized α-subunits is rapidly incorporated into tetrameric complexes with β-subunits [26]. These observations suggest that CK2 tetramer assembly is limited by the rate of CK2β dimerization. Furthermore, studies using the yeast two-hybrid system demonstrated that CK2β subunits can interact with both CK2α and CK2β, while CK2α subunits can only interact with CK2β [27–29]. Finally, we have demonstrated that complexes between two CK2β subunits can form in mammalian cells in the absence of the catalytic subunits [30]. The fact that CK2β subunits can associate in the absence of CK2α suggests that the crystal structure of dimeric CK2β is a physiologically relevant structure [31]. Here we present results demonstrating that a dimerization-deficient CK2β mutant is not capable of associating with catalytic subunits in a tetrameric complex.

Using co-immunoprecipitation assays, we demonstrated that the zinc-finger mutant CK2$\beta^{C109,114S}$ was not able to form dimeric complexes with HA-β or to form tetrameric complexes with HA-tagged CK2α' or CK2α'k.d. in mammalian cells. This conclusion was strengthened by the fact that Myc$\beta^{C109,114S}$ was not appreciably phosphorylated when co-transfected with HA-α'. In cells, the β subunit of CK2 is known to undergo autophosphorylation following tetramer assembly in an intramolecular process [23]. Since no appreciable phosphorylation of CK2$\beta^{C109,114S}$ was detected, we concluded that the zinc-finger mutant is not able to form tetrameric complexes. Furthermore, we investigated the ability of CK2$\beta^{C109,114S}$ to interact with CK2α' by examining the interactions of GST-fusion proteins with the products of *in vitro* transcription and translation. Results from this binding assay demonstrate that Myc$\beta^{C109,114S}$ was not able to stably bind to GST–CK2α'. Collectively, these results strongly suggest that the zinc-finger mutant CK2$\beta^{C109,114S}$ is not capable of forming tetrameric CK2 complexes.

Results obtained in this study using the yeast two-hybrid system indicate that the zinc-finger mutant CK2$\beta^{C109,114S}$ is not able to interact with another CK2β subunit. Co-transformation of yeast with pGBT9-CK2β and pACT2-CK2$\beta^{C109,114S}$ did not stimulate β-galactosidase expression above background levels. This result is consistent with results obtained from co-immunoprecipitation assays showing that Myc$\beta^{C109,114S}$ was not capable of dimerizing with HA-β. In addition, we observed that co-transformation of yeast with pGBT9-CK2α and pACT2-CK2$\beta^{C109,114S}$ did result in a substantial stimulation of β-galactosidase activity. This latter result from the yeast two-hybrid system is not consistent with results obtained from co-immunoprecipitation assays, CK2β-subunit autophosphorylation, or *in vitro* binding assays. We do not have a precise explanation for the discordance between the results of the yeast two-hybrid assays, and the other results obtained by co-immunoprecipitation and GST pulldowns. However, it may be noteworthy that in the yeast two-hybrid assay CK2β has been expressed as a fusion with Gal4. Since Gal4 itself dimerizes, it is plausible that Gal4-induced dimerization of CK2β allows for interaction with the catalytic subunits.

We also examined the effect of disrupting the zinc finger on protein translation rate and turnover. Using *in vitro* transcription and translation, we found that there was no effect on the translation rate of CK2$\beta^{C109,114S}$. However, studies using a protein synthesis inhibitor demonstrated that Myc$\beta^{C109,114S}$ was turned over much faster than Mycβ. We have previously shown that β-subunits are synthesized in excess of α-subunits using biosynthetic labelling experiments [26]. Moreover, this study demonstrated that β subunits that are not incorporated into tetrameric CK2 complexes are quickly degraded. The results presented here are in good accordance with this study. Our results show that a zinc-finger mutant of CK2β that is not incorporated into dimeric or tetrameric complexes is quickly degraded.

It has been shown that the C-terminal domain of the β-subunit is responsible for stable interactions with CK2α [20,30,32a]. As a result, it was somewhat unexpected that mutating cysteines 109 and 114 of the zinc finger would have an effect on α–β interactions. Furthermore, Meggio et al. [32] demonstrated that recombinant β treated with sulphydryl-modifying reagents fails to form homodimers, but forms α–β dimers and $\alpha_2\beta/\alpha\beta_2$ trimers *in vitro*. However, sulphydryl-modifying reagents would exert global effects on the cysteine residues in CK2β and CK2α, so it is not clear that these results were exclusively due to disruption of the zinc finger. Furthermore, the study by Meggio et al. [32] failed to access the possible role of post-translational modification of CK2 *in vivo*. Our results conclusively show that the zinc-finger mutant is not able to form tetrameric CK2 complexes both *in vitro* and *in vivo*.

One possible model that fits our results is that β–β dimerization induces a conformational change that facilitates interactions with the catalytic CK2 subunits. Alternatively, stable binding of the catalytic subunits may require interactions with both members of a CK2β dimer in a manner such that a monomeric form of CK2β is unable to bind catalytic CK2 subunits. Based on the fact that a dimerization-deficient CK2β mutant is not able to interact with CK2α, this model seems to be plausible.

By mutating two conserved cysteine residues in the zinc finger of CK2β, we have studied the effects of disrupting β–β dimerization on CK2 tetramer formation. Our results demonstrate that this zinc finger mutant fails to form homodimers and fails to form tetrameric complexes with CK2α. Importantly, this study also demonstrates that the formation of CK2β dimers is a prerequisite for the formation of tetrameric complexes with the catalytic subunit. Consistent with exclusion from tetrameric CK2 complexes, we have shown that Myc$\beta^{C109,114S}$ is not appreciably phosphorylated. Furthermore, we have demonstrated that Myc$\beta^{C109,114S}$ is rapidly degraded when expressed in mammalian cells. Collectively, although these studies have not yielded dominant uninhibited mutants of CK2, they further reinforce the complex nature of the interactions between the catalytic and regulatory subunits of CK2.

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