CK2α/CK1α chimeras are sensitive to regulation by the CK2β subunit

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Abstract The effect of CK2β on the activity of CK2α and other protein kinases that can bind this regulatory subunit is not fully understood. In an attempt to improve our understanding of this effect, chimeras of CK2α and CK1α have been constructed. These chimeras contain different portions of the CK2α amino terminal region that are involved in the interaction with CK2β to form CK2 tetramers. In the case of chimeras 1 and 2, the portions of CK2α replace the corresponding segments of CK1α. In the case of chimera 3, the fragment of CK2α is added to the whole CK1α molecule with the exception of the initial methionine. Chimera 3 has 8% of the activity of $CK1\alpha^{WT}$, while chimeras 1 and 2 are 3 orders of magnitude less active than $CK1\alpha^{WT}$. All three chimeras bind tightly to CK2β, but only chimeras 1 and 2 are significantly stimulated in their capacity to phosphorylate casein and canonical peptide substrates by addition of the regulatory subunit. No stimulation was observed with phosvitin or non-canonical peptides derived from β-catenin. CK2β protects chimeras 1 and 2 from thermal inactivation. Chimera 2 can phosphorylate CK2β and autophosphorylate; however, salt concentrations above 150 mM NaCl eliminate the phosphorylation of CK2β but not the autophosphorylation of chimera 2. Similarly, high salt decrease the stimulatory effect of CK2β on the phosphorylation of casein.

Abbreviations: chi1, chi2, chi3 stand for the respective chimeras.

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Introduction

Protein kinase CK2 (formally known as casein kinase II) is composed of catalytic subunits (in most tissues CK2α and CK2α′ but, CK2α″ has also been detected) and of regulatory subunits (CK2β). The holoenzyme is normally tetrameric corresponding to the composition of $(CK2\alpha)_2(CK2\beta)_2$, $(CK2\alpha)(CK2\alpha')CK2\beta_2$, or $(CK2\alpha')_2CK2\beta_2$. Larger more complex multimers of the tetramer are present at physiological salt concentrations [1–4].

The CK2α subunit is active by itself against a large number of substrates. However, in the presence of CK2β, the phosphorylating activity of CK2α is stimulated 2 to 6-fold with the majority of protein or peptide substrates. With some other substrates, such as calmodulin, the presence of CK2β completely inhibits the phosphorylating activity of CK2α [5]. With yet another set of substrates, CK2α is inactive and it requires CK2β for active phosphorylation to occur [6]. CK2β, however, has been found to have a number of different other effects on CK2 function. CK2β causes a significant increase in the thermal and proteolytic stability of CK2α [1, 7]. CK2β has also been shown to be required for the correct localization of CK2 on the internal cell membrane [8], also on its export as an ectokinase [9]. CK2β apparently furnishes "docking sites" for substrates, greatly stimulating their phosphorylation [10–13].

The elucidation by Niefind et al. [14] of the tetrameric three-dimensional structure of CK2 clarified the relationship of both subunits in these tetramers. The CK2β dimer provides the scaffold to which the two alpha subunits bind. This structure defined that the CK2α subunit region that

interacts with CK2β is comprised by only the first 73 amino acid residues out of the 392 present in the sequence of CK2α. Conversely, only the part of CK2β that includes the carboxyl 30% of that subunit participates in the interaction with CK2α. Despite the fact that these results provided extremely important information, they were puzzling because they did not readily explain the results obtained with site-directed mutagenesis or other biochemical techniques that implied interactions of the amino terminal half of CK2β with the catalytic subunits. For instance, the autophosphorylation of serine residues 2 and 3 by CK2α [15] or the inhibitory effect of the acidic cluster between amino acids 55 and 65 of CK2β on the activity of CK2α [16, 17]. Several years later, it was realized that these interactions of CK2β with the catalytic subunit were only possible due to the higher order aggregation of CK2 tetramers among themselves that brought amino terminal regions of CK2β close to the active phosphotransferyl center of the catalytic subunit [18, 19].

The regions responsible for the primary interactions between CK2β and CK2α discovered in the crystalline structure of the tetramer, however, did explain why CK2β is able to recognize other protein kinases such as A-Raf, c-mos, and Chk1. The explanation is that the region of CK2α that is bound by the regulatory subunit is part of the molecule that is significantly conserved in most kinases since it is involved in the structure responsible for ATP binding. A very interesting point in this regard is that CK2β can not only interact with these other kinases but can also regulate their phosphorylating activity. In the case of A-Raf, CK2β binding stimulated its activity in phosphorylating MEK [20]. Chk1 is also activated by CK2β in the phosphorylation of Cdc25 [21]. On the other hand, the binding of CK2β to c-mos inhibits its phosphorylating activity. In this latter case, the region of c-mos that interacts with CK2β has been defined as amino acids 52–115, which roughly coincides with the ATP binding region of the c-mos protein kinase [22].

This potential of CK2β to bind and regulate the activity of several protein kinases prompted us to postulate that CK2β might behave as a "wild card" regulatory subunit of protein kinases [11]. This capacity that transcends its activity in regulating CK2α gives a lot of added interest to the question of whether cells contain equimolar amounts of CK2 catalytic and regulatory subunits and to whether there are mechanisms that may tend to dissociate CK2β from a catalytic kinase subunit. In the case of c-mos, dissociation of this kinase from CK2β is observed upon the activation of c-mos [23].

All of these observations have led us to the question of how specific or general is the capacity of CK2β to regulate kinases. Obviously, CK2β does not regulate all kinases, but this may be due to the fact that only some kinases are able to bind CK2β tightly. We asked ourselves what if we could make a kinase that is not normally regulated by CK2β is able to bind this protein subunit. Could this kinase be regulated by CK2β?

We approached this question using protein kinase CK1α. This monomeric protein kinase shares with CK2 an affinity for targets in acidic regions of proteins, but has different canonical and non-canonical sequences that it recognizes in target substrates [24]. Together with GSK-3 and CK2, it can be primed by previous phosphorylations to recognize new substrates [25].

In the course of these experiments, we found that CK1α is not activated or inhibited by CK2β. However, CK2β is a substrate for CK1α, a fact that we are currently investigating. Aligning the catalytic domains of CK1α and CK2α, we are able to see that the region of CK2α that is responsible for binding CK2β contains two stretches of amino acids (^{41}LVRK^{44} and A^{57}INITN^{61}) that are perfectly reproduced in CK1α in residues 19–22 and 34–39. This observation suggested that it would be possible to generate chimeras of CK2α and CK1α that might contain a part or almost the total region of CK2α that is responsible for binding to CK2β, while maintaining the rest of the CK1α kinase molecule.

The experiments described here report that chimeras that fulfill those requirements are weakly active but are able to phosphorylate CK1 peptide and other protein substrates and that they bind tightly to CK2β. Furthermore, the presence of this subunit stimulates their catalytic activity very significantly with some protein and peptide substrates.

Materials and methods

Construction of chimeras and truncated CK1α

Chimeras 1, 2, 3, and CK1$\alpha\Delta$1–18 were constructed using overlap extension PCR. Platinum TaqDNA polymerase High Fidelity (from Invitrogen) was used according to the manufacturer's protocol.

Nine oligonucleotides used to generate the chimeras and the truncated CK1 were as follows:

P1. 5′ T A T A T A *G C A T G C* C G A G A C T A T T G G G A C 3′
P2. 5′ C A G A T C C G A T T T T C C T C A C T A A C3′
P3. 5′ G T T A G T G A G G A A A A T C G G A T C T G3′
P4. 5′ T A T A T A *G G T A C C* T T A G A A A C C 3′
P5. 5′ T T T A T T *G C A T G C* C T C G T T C G T A A A A T C 3′

P6. 5′ C C A T T T G T G A T G T T A A T G G C 3′
P7. 5′ G C C A T T A A C A T C A C A A A T G G 3′
P8. 5′ T T A A C A T C A C C G C C A G C A G C A G C 3′
P9. 5′ G C T G C T G C T G G C G G T G A T G T T A A 3′

Restriction enzyme sites are underlined. P1 and P5 include a *Pae I* site and P4 has a *Knp I* site. In P2, the 5′ half of the primer is complementary to CK1α and 3′ half is complementary to CK2α. In P3, the 5′ half of the primer is complementary to CK2α and the 3′ half is complementary to CK1α. In P6, the 5′ half of the primer is complementary to CK1α and the 3′ half to CK2α. In P7, the 5′ half of the primer is complementary to CK2α and the 3′ half to CK1. In P8, the 5′ half of the primer is complementary to CK2α and the 3′ half to CK1α. In P9 the 5′ half is complementary to Ck1α and the 3′ half to CK2α.

The PCR products were gel purified and digested with *Pae I* and *Kpn I* (from Fermentas) restriction enzymes. pQE 80-L vector was digested with the same enzymes and ligation of the fragments to the vector was carried out with T4DNA ligase (from Fermentas) according to the manufacturer's protocol and then transformed in the *Escherichia coli* strain BL 21 (DE 3)pLys S. All kinases were sequenced over the entire coding region (Oligonucleotide Sequencing and Synthesis Facility, University of Chile).

The initial methionine residue of the expressed chimeras and of the truncated CK1αΔ1–18, which lacks the first 18 residues in its amino terminal, is supplied by the vector pQE80-L used for cloning.

Protein expression and purification

The kinases were expressed in *E. coli* BL 21 (DE 3)pLys S cells. Cells were grown at 37 °C to an absorbance at 600 nm of 1.0. Expression of His-tagged protein was induced overnight at 20 °C in the presence of 0.1 mM isopropyl-*β*-D-thiogalactoside. Then cells were pelleted at 3,000*g* for 20 min at 4 °C and cell pellets were resuspended in buffer A (50 mM Hepes, pH 8.0 500 mM NaCl, 20% glycerol) containing 1% TritonX-100, 1 mM phenylmethyl sulfonyl Fluoride (PMSF) and 2 μg/ml each of leupeptine, aprotinin, pepstatin A, and antipain (Calbiochem). Lysozyme (1 mg/ml) (Sigma) was added and cells were lysed for 20 min on ice. Lysates were sonicated and centrifuged at 39,000*g* for 30 min at 4 °C. Supernatants were loaded onto nickel columns (Ni-NTA Purification System, Invitrogen) and affinity chromatography was done according to manufacturer's instructions. Eluted proteins were analyzed using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), specifically identified using monoclonal antibodies and densitometrically quantified using bovine seroalbumin as protein standard. The expressed enzymes used throughout were derived from : CK2α, *Xenopus laevis* [26]; CK2αD156A, *Xenopus laevis* [27]; CK2*β*, *Xenopus laevis* [28]; CK1α, *Danio rerio* [29].

Kinase activity assays

Standard reaction mixes contained 0.5–2 pmoles of enzyme, 50–200 μM ATP, [^{32}P]-γATP (Perkin Elmer) with a specific activity of 1,000–5,000 cpm/pmol in kinase buffer consisting of 50 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT in a total volume of 20 μl. Substrate used was 5 mg/ml casein or 200 μM peptide substrates. The reaction was started by the addition of ATP and stopped after 10 min at 30 °C by absorption onto phosphocellulose paper. Papers were washed three times for 30 min in 75 mM phosphoric acid, dried, and radioactivity was determined by scintillation. All assays were performed in duplicate samples. The reaction assays were submitted to analysis on 10 or 14% SDS-PAGE and analyzed by autoradiography.

Western blots

After running the reactions on 10% SDS-PAGE, the proteins were transferred to Immobilion transfer membranes (Millipore). The membranes were then incubated overnight with the primary antibody. The presence of the primary antibody was detected by a secondary antibody.

Pull down assays

Glutathione-sepharose 4B (Amersham Pharmacia Biotech AB) was mixed with 150 pmol of glutathione-s-transferase (GST) or GST-CK2*β* in a total volume of 300 μl interaction buffer containing 20 mM Hepes pH 7.5, 150 mM NaCL, 1 mM EDTA, 1 mM DTT, 1% glycerol, and 0.1% Triton X-100 and incubated for 1 h at 4 °C with gentle agitation. After 2 min of 3,000*g* centrifugation at 4 °C, the resin with bound GST-protein was washed twice with 200 μl of interaction buffer. Subsequently, the beads were incubated for 2 h at 4 °C with radioactive proteins labeled with ^{35}S using an in vitro transcription/translation system.

In vitro transcription and translation and ^{35}S-labeling of proteins

cDNA of CK1α from *Danio rerio*, cDNA of CK2α from *Xenopus laevis*, or DNA of chimeras 2 and 3 subcloned in pT7–7 or pAGA vectors were used to express proteins in

the TNT-coupled transcription/translation reticulocyte lysate assay (Promega Corp., Mason, W1). The reactions (50 µl) were performed according to the manufacturer's instructions using [^{35}S]-methionine (Amersham Biosciences Argentina, Buenos Aires, Argentina). Incubations were for 90 min at 30 °C.

Co-immunoprecipitation assays

Ten pmol of His- tagged CK1α, chimera 2 or 3 were incubated with 30 pmol of CK2β overnight at 4 °C with 2 µg of polyclonal anti-CK2β (FL-215 antibody) (Santa Cruz Biotechnology) in the same interaction buffer used in the pull down assays, and then with 20 µl of protein A Agarose (Sigma) for 4 h at 4 °C. After centrifugation at 3,000g for 1 min, the precipitates were washed five times with interaction buffer. The co-immunoprecipitated proteins were detected by Western blots probed with anti $(His)_6$ antibodies (Clontech).

Results

Construction of CK2α/CK1α chimeras

Figure 1A shows the alignment of the amino acid sequences of the catalytic kinase domains of CK2α and of CK1α in their N-terminal segments that contain all the regions of CK2α that interact with CK2β in the tetrameric crystal structure according to Niefind et al. [14]. Below, the amino acid sequences of the three chimeras constructed are illustrated. Part B of Fig. 1 shows a graphic representation of the three chimeras. The schematic representation shows that chimera 1 contains amino acids 21–44 of CK2α joined to isoleucine 23 of CK1 and the rest of that molecule. The CK2α part of this chimera includes only the first regions of this catalytic subunit that bind CK2β. Chimera 2 includes CK2α residues 21–60 joined to asparagine 39 and the rest of CK1α. This chimera contains almost all the regions of CK2α that bind Ck2β. Finally, chimera 3 contains the same CK2α portion as chimera 2 (residues 21–60), but in this case, this fragment is joined to alanine 2 of CK1α. This chimera has therefore the nearly complete sequence of CK1α, including its whole original ATP binding region.

Table 1 shows the activity obtained using casein and canonical peptide substrate with the three chimeras and with the truncated CK1Δ1–18, as compared to wild-type CK1α. CK1αΔ1–18 is a deletion mutant of CK1α that lacks the N-terminal 18 amino acids and that, therefore, reproduces the portion of CK1α that is present in chimera 1. It is evident that chimera 3 is only 8% as active as CK1α. This lower activity must be due to a negative effect of the CK2α sequence since the chimera contains practically the whole sequence of CK1α. The great loss of activity of chimeras 1 and 2 is to a large extent due to the absence of CK1α N-terminal sequence. This is shown by the fact that

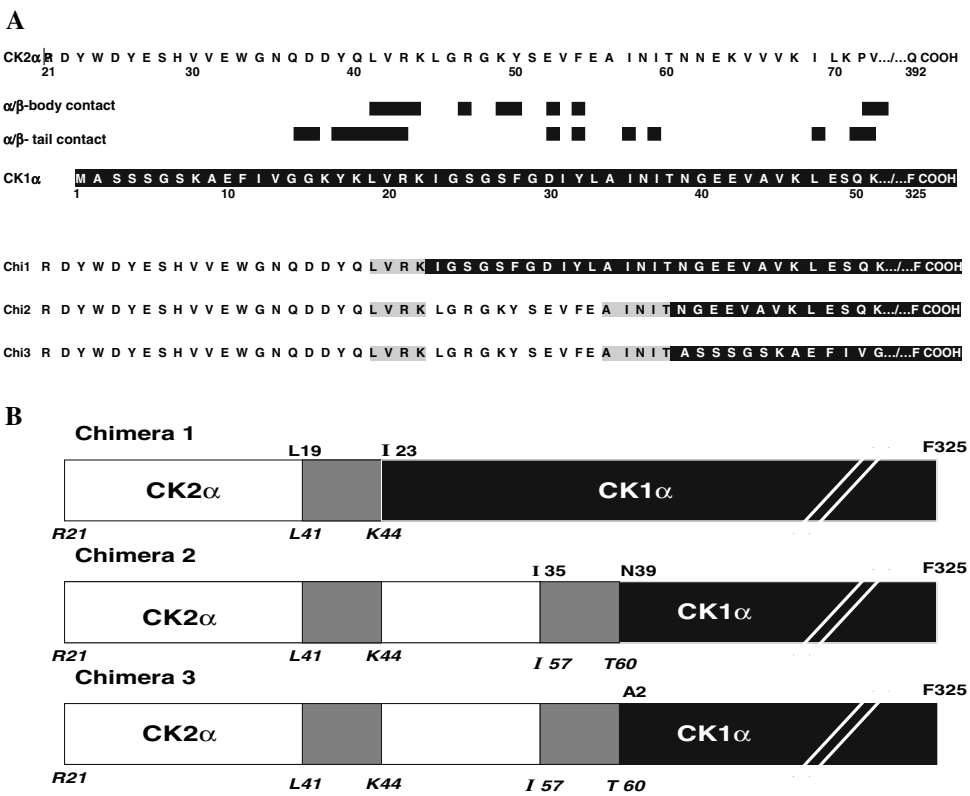

Fig. 1 Three chimeric protein kinases constructed from CK2α and CK1α. (**a**) Alignment of the N-terminal sequences of CK2α and CK1α. The body and tail contacts between CK2β subunit and CK2α subunit are shown as black bars (14) between the two sequences. Below are shown the three chimeric proteins (chimeras 1, 2 & 3) using the same alignment with the kinases. (**b**) Schematic representation of the three chimeras. Segments of the chimera corresponding to CK2α are represented in white and segments corresponding to CK1α are represented in black. Grey regions show where both kinases have the same primary sequence and the specific amino acid present in each case is indicated above the bars (CK1α) and below (CK2α)

Table 1 The phosphorylating activity of the chimeric proteins

Enzyme	Activity with Casein (pmol ^{32}P/pmol enzyme)	% of activity	Activity with CK1 canonical peptide substrate[a] (pmol ^{32}P/pmol enzyme)	% of activity
Chimera 1	0.85	0.09	7	0.7
Chimera 2	0.42	0.04	0.6	0.06
Chimera 3	73	7.8	38	3.7
CK1αΔ 1–18	0.01	<0.001	0.01	<0.001
CK1α	931	100	1,027	100

[a] R R K H A A I G D D D D A Y **S** I T A. The phosphoacceptor residue is in bold. The activity (pmol ^{32}P/pmol enzyme) was expressed for the 10 min incubation period as described in "Materials and methods"

chimera 1 is considerably more active than CK1α that has suffered deletion of the first 18 amino acids (CK1αΔ1-18). This means that the CK2α sequence added to chimera 1 has a positive effect on the activity of this protein. Comparing the activity between chimera 1 and 2, interestingly, we realize that chimera 1 is 10-fold more active with the CK1 canonical peptide substrate than with casein, which does not happen with chimera 2. The chimeras were also tested with the CK2 specific peptide but no activity was detected (not shown).

Binding of chimeras to CK2β

The binding of the chimeras to CK2β was tested by two methods: GST fusion protein pull-down assays and co-immunoprecipitation.

Figure 2 shows the results obtained using the pull-down assay. In A, GST-CK2β fusion protein and glutathione agarose beads were used to pull down the chimeras with CK2α as a positive control and CK1 as a putative negative control. The proteins tested for pull-down were labeled with ^{35}S in an in vitro transcription/translation system. Controls were also run using GST protein without CK2β fusion. There is a slight binding of radioactivity of the proteins with GST. However, more intensive bands are observed with both chimeras 2 and 3, as well as with CK2α, the positive control. With CK1α, the radioactive band obtained with GST-CK2β was only very slightly over the GST blank. Part B of the figure shows densitometric quantification after substraction of the GST blank values. Similar experiments carried out with chimera 1 showed that this protein also interacted specifically with GST-CK2β to a degree considerably higher than CK1α (not shown).

A similar experiment was run but in this case, CK2β was incubated with chimeras 2 and 3 and they were submitted to immunoprecipitation using an anti CK2β antibody (Fig. 3). The immunoprecipitates were then run on SDS-PAGE and probed with anti-His antibody because the chimeras and CK1 were all expressed in E. coli with a $(His)_6$-tag. Controlled incubations in which no antibody or

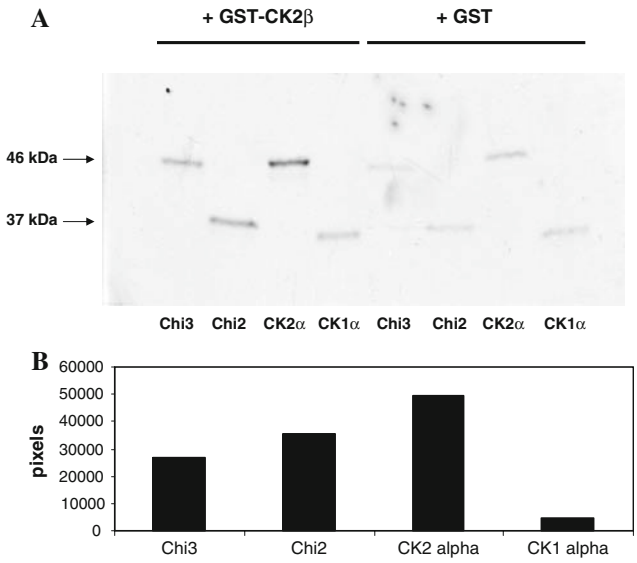

Fig. 2 (**a**) Autoradiography of the pull down assay using GST-CK2β and the ^{35}S-labeled chimeras 2 and 3 and CK2α and CK1α, subjected to SDS-PAGE as described in "Materials and methods". Controls were performed using GST (right side). (**b**) Densitometric analysis of the autoradiography in (**a**) showing relative binding capacity of the chimeras compared to CK2α and CK1α. Pixels with non-specific binding were substracted

no CK2β had been added were also run. Again chimera 2 gave a strong reaction, considerably stronger than chimera 3 and CK1α gave a slight positive effect over the blank values, where no CK2β was added. Part B of this figure again quantifies the band intensities over the blank values.

CK2β stimulates the phosphorylation activity of chimeras 1 and 2

In Fig. 4, chimeras 1 and 2 were stimulated in their phosphorylation of casein by the addition of different amounts of CK2β. In 4A, the results of the addition of CK2β are expressed as pmol of [^{32}P] incorporated to casein per min and per pmol of enzyme. In part B of the same figure, the graph reflects the fold-stimulation caused by CK2β on the activity of the chimeras tested in the absence of added CK2β. These

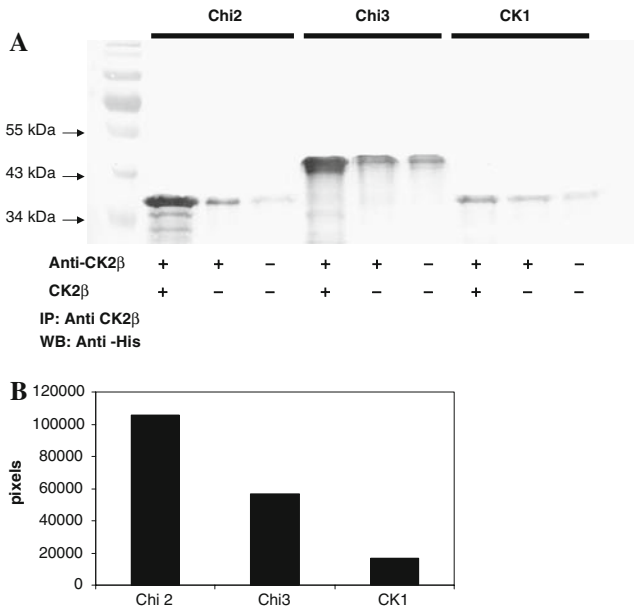

Fig. 3 (**a**) Western blot showing the co-immunoprecipitation assay between CK2β and chimeras 2 and 3. CK1α was used as a negative control and all proteins were His-tagged. The Western blot was developed with anti-His antibody as described in Methods. (**b**) Densitometric analysis of the Western blot in (**a**). Pixels with non-specific binding were substracted

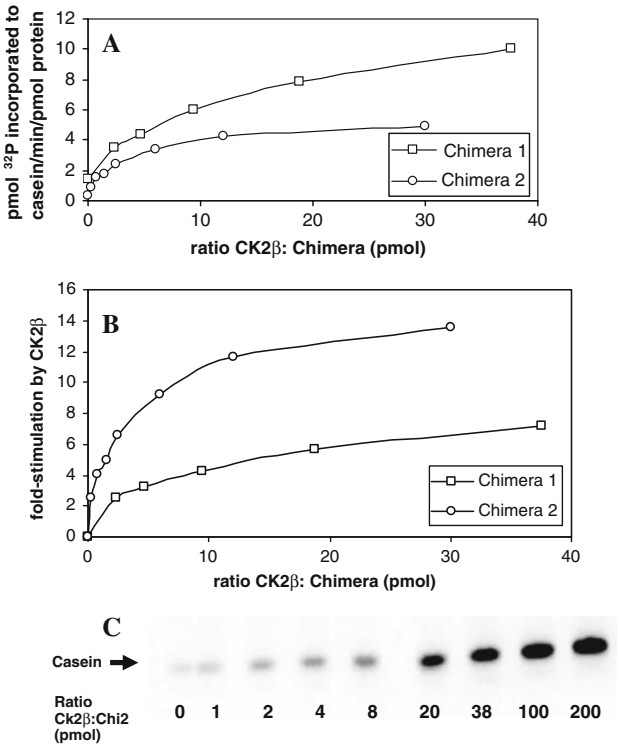

Fig. 4 Phosphorylating activities of chimeras 1 and 2 are stimulated by CK2β using casein as a substrate. (**a**) Activities of chimeras 1 and 2 were measured as described in "Materials and methods" in the presence of increasing amounts of CK2β. (**b**) Fold-stimulation by increasing amounts of CK2β of chimeras 1 and 2. (**c**) Autoradiogram showing phosphorylated casein at increasing CK2β concentrations. In vitro kinase assay of chimera 2 was performed as described in "Materials and methods" and subjected to SDS-PAGE

measurements were carried out using the phosphocellulose filter assay for activity. Comparing the curves in 4B, it can be seen that the stimulatory effect of CK2β in chimera 2 is considerably higher than the effect obtained with chimera 1. It can also be observed that with chimera 2, lower concentrations of CK2β are required to obtain near maximum effects, as compared with the results obtained with chimera 1. This is what could be expected from the fact that chimera 2 has a longer segment of CK2α containing the region of interaction with CK2β. Similar experiments carried out with chimera 3 demonstrated no detectable effect of CK2β on the activity of this chimeric protein (not shown). In 4C, on the other hand, it is possible to see the effect of CK2β in an autoradiography of SDS-PAGE in which the increment in the radioactivity incorporated into casein is catalyzed by chimera 2 with increasing amounts of CK2β.

We have also obtained evidence demonstrating that the stimulatory effect caused by addition of CK2β to these chimeras is due to the binding of the regulatory subunit to the CK2α region they possess. This evidence was obtained by allowing chimera 2 and CK2α to compete for the beta subunit. In Fig. 5a, we see the effect of a limiting amount of CK2β on the phosphorylation of a canonical peptide substrate for CK1. The stimulation caused by CK2β is approximately 2-fold (sample 2 as compared to sample 1). If we introduce CK2α^{D156A} into these assays which is catalytically inactive but tightly binds CK2β [27], we see that the presence of increasing amounts of this subunit causes a gradual reduction in the stimulation caused by CK2β (samples 3–6). A similar observation can be obtained with CK2α phosphorylating a specific CK2 peptide substrate as analyzed by autoradiography of the peptide substrate (Fig. 5b). The stimulation of the phosphorylation caused by CK2β (comparing sample 2 to sample 1) is gradually reduced by addition of chimera 2, which competes with CK2α for the limiting CK2β subunit (samples 3–5). Controls with chimera 2 and this CK2 peptide substrate demonstrated that the chimera cannot phosphorylate this peptide.

Apparent Km

Attempts were made to determine the apparent Km for chimeras 1 and 2 in the presence and absence of CK2β. Chimera 1 showed an anomalous behavior with a bimodal curve for the Lineweaver-Burke plot when using ATP as a substrate. This made the determination of the effect of CK2β on this parameter difficult. The determination of the apparent Km for ATP and casein for chimera 2 yielded typical results (Table 2). It can be observed that the app. Km for ATP of the chimera 2 is higher than that for CK1α

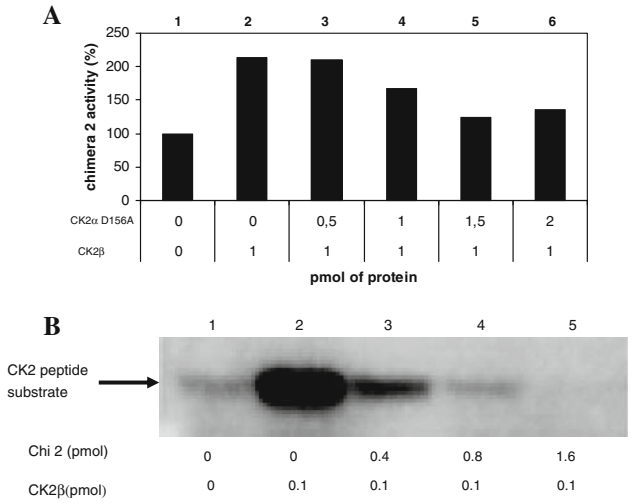

Fig. 5 (**a**) Competition for CK2β between chimera 2 and catalytically inactive mutant CK2α^{D156A} (26). The in vitro kinase assay of 0.7 pmol of chimera 2 was measured as described in "Materials and methods" using as substrate canonical CK1 peptide R R K H A A I G D D D D A Y S I T A, adding CK2β simultaneously where indicated and increasing amounts of dominant negative CK2α^{D156A}. Arbitrarily, we assigned 100% to the activity measured with the chimera 2 without CK2β. (**b**) Competition for CK2β between chimera 2 and CK2α^{WT}. The in vitro kinase assay of 0.05 pmol of CK2α^{WT} was measured without and with CK2β and in the absence or presence of chimera 2, which was added simultaneously in increasing amounts. The specific peptide substrate for CK2: R R A D D S D D D D D was used. The reactions were analyzed using 14% SDS-PAGE and the autoradiogram shows the phosphorylated peptide

Table 2 Apparent Km values for ATP and Casein

Enzyme	App. Km (ATP) (μM)	App. Km (Casein) (mg/ml)
CK1α	33	1.2
CK2α	15	1.0
Chimera 2	90	0.5
Chimera 2 + CK2β	24	0.6

Comparison of the apparent Km of Chimera 2 for ATP and Casein in the presence and absence of CK2β and with the wild-type CK1α and CK2α

or CK2α. The presence of the CK2β is able to lower this value to a near normal value for the wild-type parent enzymes. Chimera 2 has very similar values for the app. Km for casein and the presence of CK2β does not significantly change this value.

Effects of CK2β on the phosphorylation of different peptides and protein substrates by chimeras 1 and 2

Table 3 shows the response of chimeras 1 and 2 to CK2β when assayed against different protein and peptide substrates.

It can be seen that CK2β has a very significant effect when casein and CK1 canonical peptides are used as substrates. No difference is observed when previously phosphorylated canonical peptides are used. This holds true for both chimera 1 and chimera 2. Chimera 2, however, is much less active in the phosphorylation of the peptide substrates than chimera 1. On the other hand, neither chimera can be stimulated by CK2β to phosphorylate phosvitin, which is a very poor substrate with chimera 2.

The β-catenin peptide that surrounds CK1 target serine 45 in that protein and that is non canonical substrate of CK1 [23] is phosphorylated by the chimeras, albeit very poorly by chimera 2. These phosphorylations are not stimulated significantly by CK2β.

High concentrations of salt block the stimulatory effect of CK2β and its phosphorylation by chimera 2

When very high specific activity of [^{32}P]-γ ATP is used (50,000 cpm/pmol), it is possible to see the autophosphorylation of chimera 2 and also the phosphorylation of added CK2β (Fig. 6a). If the concentration of salt is increased, the phosphorylation of CK2β is inhibited and practically disappears above 150 mM. The same salt concentrations, on the other hand, do not significantly affect the autophosphorylation of chimera 2 itself.

In a similar fashion, the stimulatory effect of CK2β on the phosphorylating activity of chimera 2 on casein is also affected by increased salt concentration until it is eliminated at 500 mM salt (Fig. 6b). These high salt concentrations do not affect the phosphorylation of casein by chimera 2 in the absence of CK2β.

CK2β protects the chimeras against thermal inactivation

One of the effects of CK2β on CK2α is that the presence of the regulatory subunit causes a very significant increase in the stability of the enzymatic activity against thermal inactivation [7].

Figure 7 shows that the chimeras 1 and 2 are very labile and suffer a rapid loss of activity when incubated at 36 °C. The addition of CK2β causes a significant stabilization of these chimeras. The effect of CK2β on chimera 2 is more pronounced than that observed with chimera 1. Controls in which BSA was added to the chimera solution at protein concentrations equivalent to those of CK2β did not protect against thermal inactivation (not shown).

Chimera 3 and CK1α were much less labile than chimeras 1 and 2 and the addition of CK2β had no appreciable effect on the inactivation at 40 °C (not shown).

Table 3 Stimulation of CK2β on the activities of Chimera 1 and Chimera 2 with different substrates

Substrate (concentration)	Activity (pmol ^{32}P/min)		Fold stimulation	Activity (pmol ^{32}P/min)		Fold stimulation
	Chi1	Chi1 + CK2β		Chi2	Chi2 + CK2β	
Casein (5 mg/ml)	0.7	2.1	3.0	0.35	2.2	6.3
Phosvitin (4 mg/ml)	0.34	0.2	–	0.04	0.03	–
CK1canonical peptide substrate[a] (200 μM)	0.75	2.1	2.8	0.06	0.25	4.2
CK1canonical phosphorylated peptide substrate[b] (200 μM)	0.7	1.9	2.7	0.02	0.08	4
CK1 non canonical peptide substrate[c] (β- catenin) (200 μM)	0.3	0.4	1.3	0.03	0.03	0

[a] R R K H A A I G D D D D A Y **S** I T A

[b] R R K H A A I G p**S** A Y S I T A

[c] R R R G A T T T A P **S** L S G K G N P E D E D V D T N Q V L Y E

The phosphoacceptor residues are in bold. The pS denotes phosphoserine. All peptides were kindly facilitated by Dr. L. Pinna

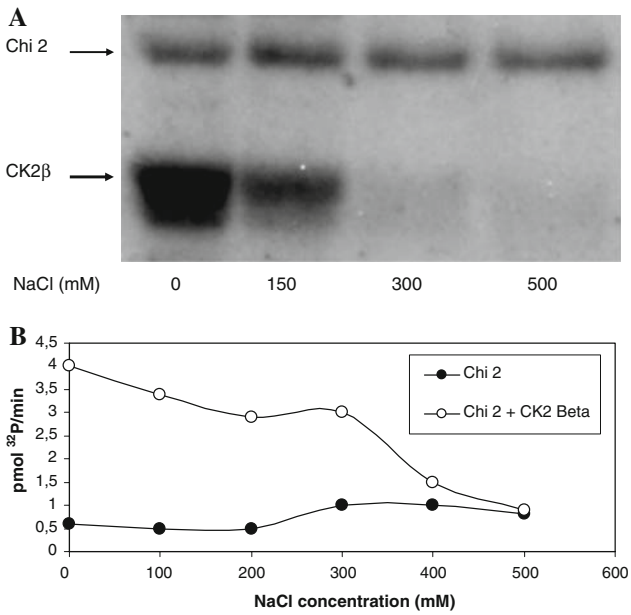

Fig. 6 (**a**) Autoradiogram of the effect of NaCl concentration on the phosphorylation of CK2β and the autophosphorylation of chimera 2. The in vitro kinase assay under increasing concentration of NaCl was performed as described in "Materials and methods", except that the reaction was carried out for 30 min and with specific activity of ATP of 50,000 cpm/pmol. In this assay, 5 pmol of chimera 2 and 10 pmol of CK2β were used. The autoradiograph was exposed for 22 h. (**b**) Kinase activity measured under standard conditions using casein as a substrate with increasing amounts of NaCl

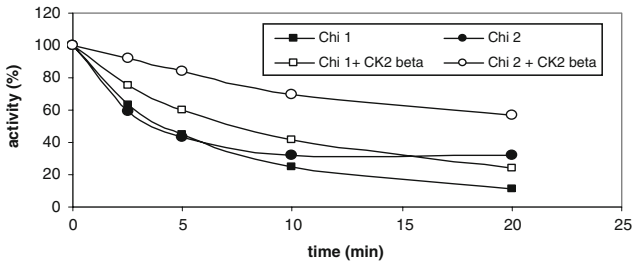

Fig. 7 Thermal inactivation of chimeras 1 and 2 at 36 °C and protection by CK2β. Thermal inactivation was performed incubating 1 pmol of chimeras + 20 pmol CK2β for 0, 2.5, 5, 10 and 20 min at 36 °C in the absence or presence of CK2β. The kinase assay was performed using the conditions described in "Materials and methods". Arbitrarily, we assigned 100% to the activity measured with the chimeras without preincubation

Discussion

The large family of protein kinases share common features in the structure of their catalytic domains and yet vary greatly with regard to protein substrate specificity, manner of regulation, binding to protein complexes, and cellular localization. It is somewhat surprising, therefore, that chimeras of different kinases, which can be easily engineered with recombinant DNA technology, have been sparingly used in studies of kinase structure and function. One notable example of construction of chimeras combining fragments of two different protein kinases was reported by Liu et al. [30], in which the mutated ATP binding site of v-Src was used to replace the corresponding region of c-Abl. The chimeric kinase kept the unusual specificity for ATP derivatives of v-Src and yet recognized the normal c-Abl protein substrates. Other examples of studies involving protein kinase chimeras involve the MAP or ERK kinase in which the main amino and carboxyl lobes of this sub-family of kinases were exchanged [31].

In this study, we have used a similar strategy to generate CK2α/CK1α chimeras that are able to bind tightly to the CK2β regulatory subunit but maintain the peptide substrate specificity of CK1.

One of the chimeras (chimera 3) merely fused a 40 amino acid region of CK2α that contains approximately 80% of the structure of this protein that binds CK2β to the second amino acid of CK1α. This chimera, which had about 8% of the activity of WT CK1α, binds CK2β strongly, but the addition of this regulatory subunit does

not significantly regulate the phosphorylating activity of the chimera.

In the construction of the other two chimeras, the part of the CK2α that was introduced replaced the equivalent region of CK1α. In the case of chimera 1, a 20 amino acid stretch of CK2α that contains about 40% of the region interacting with CK2β was jointed to L19 of CK1α. In addition, in the case of chimera 2, the same 40 amino acid stretch of CK2α, as used in chimera 3, was fused to N39 of CK1α. Chimeras 1 and 2 were 3 orders of magnitude less active than $CK1\alpha^{WT}$. This loss of activity appears to be mainly due to the elimination of N-terminal residues of CK1α that are required for activity, as is shown by the fact that CK1αΔ1-18, which lacks the same portion of CK1α as does chimera 1, is practically inactive. This observation would indicate that the presence of the CK2α N-terminal region in chimera 1 can help restore some activity to this kinase. An important observation, however, is that the chimeric kinases maintain the substrate specificity of CK1α toward peptides, which is in agreement with the observation that the recognition of these substrates depends mostly on the carboxyl terminal half of the kinase domains [32]. It is not surprising that the apparent Km for ATP of the chimeras is affected with respect to the wild-type enzymes since the construction of these proteins disrupts the nucleotide binding pocket.

The striking result obtained in this work is the finding that the addition of CK2β to chimeras 1 and 2 can cause a very significant stimulation of their phosphorylation activity with substrates such as casein and CK1 specific canonical peptide substrates. The effect of CK2β depends on the amount of the regulatory subunit added and as expected, the stimulation of chimera 2 that has the larger region binding CK2β is more pronounced and requires lower amounts of CK2β. The competition experiments in which CK2α and chimera 2 compete for a limiting amount of CK2β, confirms the hypothesis that the stimulation of phosphorylation of both of these kinases is due to the binding to the same equivalent region of the enzyme that occurs with CK2α. The fact that chimera 3, which binds CK2β, is not stimulated by the addition of this subunit indicates clearly that the binding itself is not sufficient to stimulate the activity, but that the location of the binding region with respect to the architecture of the molecule is a key factor in the regulation.

The binding of CK2β also causes an increase in the thermal stability of the chimeras, especially chimera 2. This is similar to the effect of the regulatory subunit on CK2α.

Studies with chimera 2 in the phosphorylation of casein show that the presence of CK2β causes a reduction in the apparent Km for ATP. This effect, however, is not sufficient to explain the total stimulation caused by the regulatory subunit, because assays that show the stimulation were carried out at 200 μM ATP, which is more than 2-fold over the app. Km.

The stimulatory or inhibitory effect of CK2β on the phosphorylating activity of CK2α is also dependent on the protein or peptide substrates used [13]. The results obtained here indicate a similar property for the chimeras 1 and 2. Stimulation by CK2β is observed with casein and with the specific canonic peptides but not with phosvitin or with the non-canonical peptide derived from β-catenin. Evidence has been obtained that demonstrates that CK1α recognizes canonical and non-canonical substrates using different structural determinants [33]. It is possible, therefore, that CK2β might selectively affect one type of substrate and not the other. The low phosphorylating activity of the chimeras makes the study of full length proteins that are natural substrates for CK1 difficult, but this question is presently being studied.

One of the most interesting observations reported here is the effect of salt on the stimulatory effect of CK2β on chimera 2 and also its effect on CK2β phosphorylation. As observed in Fig. 6, CK2β can be phosphorylated by chimera 2. This phosphorylation can only be detected using very high specific activity ATP. The autophosphorylation of the chimera can also be detected. The phosphorylation of CK2β, however, turns out to be very sensitive to salt. When salt goes over 150 mM, the phosphorylation of CK2β practically disappears, while no great effect is observed on the autophosphorylation of the chimera 2.

The capacity of CK2β to stimulate the phosphorylation of casein mediated by chimera 2 is also highly dependent on salt. The stimulation disappears at 500 mM salt. Salt, on the other hand, has very little effect on the activity of the chimera alone. The interesting aspect of this is that this effect of salt closely resembles the observations with CK2 and is related to the capacity of CK2 tetramers to form aggregates that allow amino-terminal portions of CK2β to approach the catalytic centers of CK2α. The observations on the effect of salt on the autophosphorylation of CK2β by CK2α demonstrated by Pinna's laboratory [18] closely resemble the results presented here. The results in Fig. 6B, which show that high salt eliminates the stimulation of chimera 2 by CK2β while having little effect on the activity of the free chimera, suggest that the stimulation caused by the regulatory subunit would be due to the formation of aggregates of tetramers involving the chimeras and CK2β. Obviously, this hypothesis requires future validation.

Various studies have been reported that demonstrate that CK2β binds directly a number of CK2 substrates. It has been proposed that some of the regulatory effects of this subunit on these substrates would be due to the presence of these "docking sites" that would bring substrate molecules

near to the catalytic subunits [10–13]. Chimeras such as the ones described here that can tightly bind to CK2β can test this proposal by determining whether protein substrates that interact with this subunit become better substrates for the chimeras.

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