Site-directed mutants of the β subunit of protein kinase CK2 demonstrate the important role of Pro-58

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Abstract The following amino acids of the *Xenopus laevis* β subunit of protein kinase CK2 (casein kinase 2) were changed to alanine: Pro-58 ($\beta_{P\rightarrow A}$); Asp-59 and Glu-60 and Glu-61 ($\beta_{DEE\rightarrow AAA}$); His-151–153 ($\beta_{HHH\rightarrow AAA}$). The last 37 amino acids of the carboxyl end were deleted ($\beta_{\Delta 179-215}$). Stimulation of CK2α catalytic subunit activity was measured with casein as substrate and the following relative activities were observed: $\beta_{P\rightarrow A} > \beta_{DEE\rightarrow AAA} \gg \beta_{WT} > \beta_{HHH\rightarrow AAA} \gg \beta_{\Delta 179-215}$. The $\beta_{DEE\rightarrow AAA}$ and $\beta_{P\rightarrow A}$ were similar to β_{WT} in reducing CK2α binding to DNA but $\beta_{\Delta 179-215}$ was less active. The results indicate that both Pro-58 and the surrounding acidic cluster play roles in dampening the activation of CK2α and that the carboxyl end of β is involved in the interaction with CK2α.

Key words: Casein kinase 2; Protein phosphorylation; *Xenopus laevis*

1. Introduction

Protein kinase CK2 (also known as casein kinase 2) is ubiquitous in eukaryotic cells and is located in both the nucleus and cytoplasm. This enzyme is responsible for the phosphorylation of more than 100 protein substrates [1–3]. CK2 is present in cell extracts as a heterotetramer with a structure $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$, in which the analogous α and α′ subunits contain the catalytic center and are active by themselves. The β subunits are regulatory since they can cause a 5–10-fold increase in the phosphorylating activity of α or α′ with most protein substrates. However, with a few proteins such as calmodulin the β subunit inhibits their phosphorylation by CK2α [4]. In addition to a regulation of the catalytic activity, the β subunit stabilizes CK2α against thermal and proteolytic inactivation [5] and decreases its binding to DNA [6].

Bacterial and baculovirus expression of cloned cDNA coding for both subunits of CK2 from several species have been reported [7–9]. This achievement, added to the fact that the recombinant CK2α and β subunits can combine with each other to reconstitute a fully active holoenzyme in vitro, has opened the way to experiments of site-directed mutagenesis in order to explore the structure and function relationship of both enzyme subunits [10–12].

The laboratories of Issinger and Pinna have collaborated in a series of experiments that have provided relevant information about the effects of a large number of mutations of the β subunits [13,14]. Their main conclusions can be summarized as follows:

(1) The acidic cluster (amino acids 55–64) of the β subunit are responsible for a negative control of enzyme activity. This conclusion is based on the fact that mutations of the acidic residues in these positons to alanine result in β subunits that stimulate the CK2α subunit phosphorylating activity to a greater extent than the wild-type β. The same acidic stretch also influences the autophosphorylation of the β subunit which occurs at Ser-2 and Ser-3. In addition, mutants altered in this region were found to have lost their capacity to inhibit the phosphorylation of calmodulin by the α subunit of CK2.

(2) Amino acids 171–181 of β are involved in its interaction with CK2α and residues 181–208 are also involved in the formation of the holoenzyme.

In our laboratory, we have studied the protein kinase CK2 obtained from *Xenopus laevis*. The cDNAs coding for the CK2α and β subunits have been cloned [15] and expressed in *E. coli* and the effects of mutations of the β subunit in the autophosphorylation site [16] and of the highly basic region of CK2α have been reported [17].

In this communication we present the results obtained with 4 different mutations of the *Xenopus laevis* CK2β subunit. The results obtained confirm and extend the findings of Boldyreff et al. [13,14] about the importance of the acidic region (amino acids 55–64) in dampening the overall activation caused by β and about the role of the carboxylic end region in determining the affinity of β for the CK2α subunit.

In addition, results are presented that demonstrate that Pro-58 which is in the middle of this same acidic region also constitutes an important structural feature in that it affects its down-regulation function with respect to the catalytic activity of CK2α. Mutation of this proline to alanine produces a hyperactive β subunit, similar to that obtained by mutation of the acidic residues alone. In addition to the effects on the catalytic activity of the reconstituted holoenzyme, the β mutants also were assayed with respect to their capacity to decrease the binding of CK2α to DNA, with respect to inhibition by a random copolymer of glutamic acid and tyrosine and to the thermal stability of the enzyme.

2. Materials and methods

2.1. Clones of CK2α and CK2β and their expression

The recombinant cDNA clones coding for the CK2α and CK2β subunits from *Xenopus laevis* were the same as those previously described [15] and their expression in bacteria and subsequent purification to obtain the recombinant proteins was also as reported [16].

2.2. Preparation of mutants

Four different mutants were prepared using PCR amplification of fragments of the cDNA for the CK2β subunit cloned in the pGEX-2T vector. The bases underlined in the primer sequences indicate the changes introduced in the cDNA sequences.

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$\beta_{\Delta 179\text{-}215}$. This deletion mutant was prepared using as primers for the PCR reaction: 5′-GGCGACCATCCTCCAAAA-3′ (primer 1) which corresponds to the 5′ of the cloning site of the vector pGEX-2T and 5′-GGGGAATTCACCTCTTGGGCCTATA-3′ (primer 2) which contains a termination codon complement (underlined) after the triplet coding for Arg-178. After amplification, the PCR product was purified, cut with *Eco*RI and subcloned again in the pGEX-2T vector.

$\beta_{P\rightarrow A}$. A mutant in which Pro-58 was changed to alanine was prepared by the mega primer method [18]. The first amplification by PCR used as primers: 5′-GGATCTAGAAGCTGATGAGGA-3′ (primer 3) in which the proline codon is changed to an alanine codon and 5′-CAGATCGTCAGTCAGTC-3′ (primer 4) corresponding to the 3′-site of subcloning in the pGEX-2T vector.

The product of this amplification reaction was purified on a DEAE membrane and used itself as a mega primer together with primer 1 in a second PCR amplification to give the full-length mutated DNA, which was digested with *Eco*RI and inserted into the pGEX-2T vector.

$\beta_{HHH\rightarrow AAA}$. The mutant in which His-151, -152 and -153 were replaced by alanines was prepared by overlapping fragment extension PCR method [19], using 3 amplification reactions. The first amplifications used 5′-AATCCTCACGGGCTGCTGCCACCGATGGAG-3′ (primer 5) in which the underlined bases are changed to convert histidine codons to alanine codons, and primer 4 as described above. The second amplification used 5′-CTCCATCGGTGGCAGCAGCCCG TGAGGATT-3′ (primer 6) which is complementary to primer 5 and primer 1 to permit the amplification of the fragment coding the amino end of the β subunit. Finally the products of these two amplifications were mixed and a third PCR was run in the presence of primers 1 and 4 to yield the full-length mutated cDNA for $\beta_{HHH\rightarrow AAA}$ which was purified, digested with *Eco*RI and inserted into pGEX-2T.

$\beta_{DEE\rightarrow AAA}$. The mutant in which Asp-59 and Glu-60 and -61 were replaced by alanines. The PCR reactions [19] were carried out with primers: 5′-ATTATCTTCCAATGCCGCAGCAGGTTCTAGA-3′ (primer 7) and primer 1 to yield the fragment coding the amino end and the overlapping fragment was amplified with primer 5′-TC-TAGAACCTGCTGCGGCATTGGAAGATAAT-3′ (primer 8) and primer 4 to produce the mutated fragment coding the carboxyl end. The two overlapping fragments were mixed and the final amplification was carried out in the presence of primers 1 and 4 to yield the complete mutated $\beta_{DEE\rightarrow AAA}$ cDNA which was purified, digested as before and inserted into the pGEX-2T vector.

All the mutants were completely sequenced by the Sanger method [20] to verify that no other mutations had been introduced by the PCR amplification method.

2.3. Assay for the phosphorylation activity of CK2α and the reconstituted holoenzyme

The kinase activity of CK2α and of the holoenzyme reconstituted by addition of either β_{WT} or 1 of the 4 different β mutants was assayed essentially as reported previously using casein as a substrate [16]. Briefly the incubation mixture (50 μl) contained: 50 mM HEPES, pH 7.8; 100 mM NaCl; 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 5 mg/ml casein, and 50 μM γ[^{32}P]ATP (500–1000 cpm/pmol). Incubations were carried out for 10 min at 30°C and the product was analyzed by absorption to Whatmann P81 paper that was washed 3 times in 75 mM phosphoric acid. All assays were carried out in duplicate.

2.4. Assay of the effect of the β subunit on the binding of [^{32}P]DNA to the α subunit of CK2

The assay for the retention of the CK2α [^{32}P]DNA complex on nitrocellulose membranes and the effect of the addition of the β subunit were carried out as described [6]. The [^{32}P]DNA used was the 568 bp DNA fragment of human DNA corresponding to an upstream region of the CK2β gene [21] which binds non-specifically to CK2α from *Xenopus* [6]. This fragment was prepared by PCR amplification using radioactive α[^{32}P]dATP as described in that report.

Incubations were carried out in 35 μl and contained 40 mM HEPES pH 7.8; 50 mM KCl; 5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM dithiothreitol; 2–10 pmol of recombinant purified CK2α and [^{32}P]DNA (50,000–200,000 cpm) at the concentrations specified. The reaction was started by the addition of the enzyme and incubation was for 5 min at 0°C. The reaction was stopped by dilution with 1 ml of cold assay buffer and immediately filtered through Millipore filters which were washed twice with 1 ml of the same buffer. When CK2β or its mutants were added, the CK2α and β subunits were preincubated for 1 h at 0°C in order to allow interaction of these subunits prior to their incubation with DNA. Analyses were performed in duplicate and corrections were made for radioactivity observed in the absence of added CK2α.

2.5. Reagents

Oligonucleotide primers were synthesized in an Applied Biosystems instrument by Oligopeptido-Chile, the Core facility of the University of Chile.

Radioactive nucleotides were from ICN. Random copolymer(Glu:Tyr) (4:1; 46,000 MW average), dephosphorylated β-casein, dithiothreitol, and buffers and salts were from Sigma Chemical Co. Nitrocellulose filters (HA 0.45 μm) were obtained from Millipore.

3. Results

The mutant β subunits were assayed for their capacity to activate the phosphorylating activity of the α subunit of CK2 using casein as substrate. The results shown in Fig. 1 confirm previous results [16] that the wild-type β subunit stimulates the activity of CK2α approximately 5-fold at nearly stoichiometric concentrations. The $\beta_{DEE\rightarrow AAA}$ and $\beta_{P\rightarrow A}$ mutants, on the other hand, cause a stimulation of 9–10-fold, roughly twice the activity observed with the β_{WT}. The mutant $\beta_{HHH\rightarrow AAA}$ is somewhat less active than the β_{WT} and $\beta_{\Delta 179\text{-}215}$ gives little stimulation at the concentrations tested. Since β subunit preparations show a decrease in stimulatory capacity upon storage, 3 separate preparations were tested giving results consistent with the differences in activity shown in Fig. 1. Previous results of Boldyreff et al. [13,14] had demonstrated that the carboxyl terminal portion of CK2β was important for its interaction with CK2α subunit. For that reason in the present study the activity of CK2 $\beta_{\Delta 179\text{-}215}$ was assayed at a very high molar excess over CK2α. Fig. 2 shows that using a 50-fold excess of $\beta_{\Delta 179\text{-}215}$ a 3-fold stimulation of CK2α activity can be obtained.

The direct interaction of CK2α and β subunits was also tested with sucrose gradient sedimentation which can differentiate the CK2α monomeric form from the $\alpha_2\beta_2$ tetramer. This assay showed that the $\beta_{DEE\rightarrow AAA}$, $\beta_{P\rightarrow A}$ and the $\beta_{HHH\rightarrow AAA}$ mutants were able to form a tetramer complex with CK2α, similar to that observed with β_{WT}.

The $\beta_{\Delta 179\text{-}215}$, however, had to be added at 20-fold excess over CK2α in order to observe a shift in the sedimentation of the catalytic activity to the position of the faster sedimenting tetramer (not shown).

Recently, we have observed that the β subunit greatly diminishes the capacity of the CK2α subunit to interact with DNA [6]. This finding provides a different assay to test the interaction

Table 1
Inhibitory capacity of copoly(Glu:Tyr) (4:1) with CK2 reconstituted with different mutants of the β subunit

CK2 subunits present	Copoly(Glu:Tyr) (4:1) (I_{50}, nM)
α	56
$\alpha + \beta_{WT}$	10
$\alpha + \beta_{P\rightarrow A}$	9
$\alpha + \beta_{DEE\rightarrow AAA}$	7
$\alpha + \beta_{HHH\rightarrow AAA}$	6
$\alpha + \beta_{\Delta 179\text{-}215}$	31

Phosphorylation of casein by CK2 was assayed as described in Methods, 2 pmoles of purified recombinant CK2α and where indicated 4 pmoles of purified recombinant β_{WT} and mutants were added. Copoly(Glu:Tyr) (4:1) was added at 8 different concentrations from 2.5 to 120 nM for the I_{50} determination.

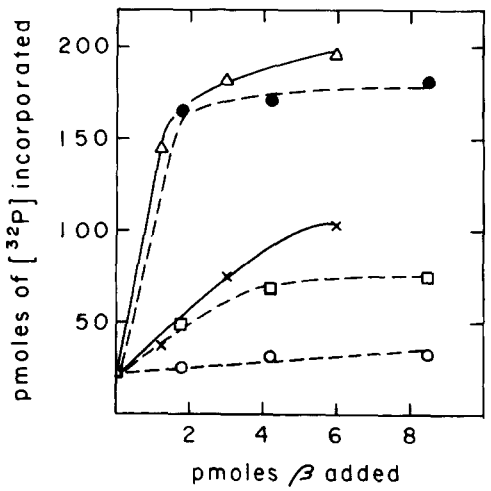

Fig. 1. The effect of mutant β subunits on the phosphorylation of casein by CK2α. The assay of phosphorylation of casein by CK2α was carried out as described in Methods using 3 pmoles of purified recombinant CK2α and the specified amount of β subunits: ×—×, β_{WT}; ● – – – ●, $\beta_{DEE \rightarrow AAA}$; △—△, $\beta_{P \rightarrow A}$; □ – – – □, $\beta_{HHH \rightarrow AAA}$; and ○ – – – ○, $\beta_{\Delta 179-215}$.

of the CK2α and β subunits. Fig. 3 shows the results obtained with 3 different β mutants when the CK2α-dependent retention of [^{32}P]DNA on nitrocellulose membranes was tested. In contrast to what was observed with the phosphorylation assay, the DNA retention assay demonstrated that the $\beta_{DEE \rightarrow AAA}$ and $\beta_{P \rightarrow A}$ is as effective as the β_{WT}. In the case of the $\beta_{\Delta 179-215}$ mutant the results obtained with this assay are coincident with the phosphorylation assay since this deleted subunit was also found to be much less active than the β_{WT}. However, a 3-fold excess of $\beta_{\Delta 179-215}$ over CK2α, a condition that had very little effect in the phosphorylation assay, caused a 50% reduction in the retention of DNA by CK2α.

Another well defined effect of the β subunit on CK2α is its capacity to change in the specificity of the phosphorylating activity towards substrates and inhibitors [4]. One example of such an effect is provided by the finding that the random polypeptide copoly(Glu:Tyr) (4:1) is a much stronger inhibitor of the $\alpha_2\beta_2$ holoenzyme than of the isolated CK2α subunit [16].

Table 1 shows the change in I_{50} values for copoly(Glu:Tyr) (4:1) observed upon the reconstitution of the holoenzyme using different β mutants at a ratio of 2:1 β/α. It is again observed that all the mutants with the exception of $\beta_{\Delta 179-215}$ cause a similar reduction of the I_{50} values of this inhibitor.

The mutants were also tested for their effect in causing the thermal stabilization of the catalytic activity. In this analysis, again, all the mutants with the exception of $\beta_{\Delta 179-215}$ can efficiently reduce the inactivation of CK2α at 45°C (not shown).

4. Discussion

The results presented above confirm and extend our knowledge about some of the structural features of the β subunit of protein kinase CK2 that are relevant to its capacity to modify the properties of the catalytic subunit of this enzyme. As previously described by Boldyreff et al. [13,14] our results confirm the observation that the acidic amino acids in positions 55–64 are important in the effect of β on the catalytic activity of CK2α. In agreement with their results, it is found that a triple mutation of Asp-59 and Glu-60 and -61 to alanine produce a β subunit that is 2-fold more active than the β_{WT} in stimulating the kinase activity of CK2α. Interesting new information is added by the finding that mutation of Pro-58, which is immersed in this acidic cluster, to alanine has a similar effect as the mutations of the acidic amino acids, resulting in a hyperactive β subunit. This observation suggests that the polyanionic nature of this region is not the only relevant characteristic in the dampening of the activation of the phosphorylating activity of CK2α. It is well known that proline residues have pronounced effects on the conformation of peptides and proteins, inducing turns in their secondary structure. In this regard, it is relevant that 25 out of 105 natural protein sequences, known to be phosphorylated by CK2, contain proline residues that are proximal on the amino end to the serines and threonines that accept the phosphoryl group from the enzyme [22]. As required by the specificity determinants of CK2, those proline residues are followed towards the carboxyl end by highly acidic clusters of amino acids. This analysis suggests that a structural turn followed by an acidic cluster may favor the interaction of this region with the peptide substrate site of the α subunit of CK2. The acidic region between amino acids 55 and 64 of the β subunit may be dampening the activation of CK2α by providing an inhibitory pseudosubstrate structure. It would be important to complement these experiments in the future with an assay of the effect of the CK2$\beta_{P \rightarrow A}$ mutant on the phosphorylation of calmodulin by CK2α as has been done by Meggio et al. (23) with the mutants lacking the neigboring acidic residues.

It had been observed [13,14] that deletion of the last 35 amino acids yielded β subunits ($\beta_{\Delta 181-215}$) that were less active than the wild-type but at a ratio β/α of 2:1 caused a full 5-fold activation of CK2α kinase activity while a deletion of 45 amino acids ($\beta_{\Delta 171-215}$) yielded a completely inactive enzyme. The $\beta_{\Delta 179-215}$ mutant studied in this work falls in between these two extremes since its affinity is more than 10-fold lower than that of $\beta_{\Delta 181-215}$, requiring up to a 50 β/α ratio to attain good activation and a ratio of 20:1 to shift the sedimentation of CK2α in a sucrose gradient. It is interesting that the two amino acids absent in $\beta_{\Delta 179-215}$ and present in $\beta_{\Delta 181-215}$ include again a proline (P180).

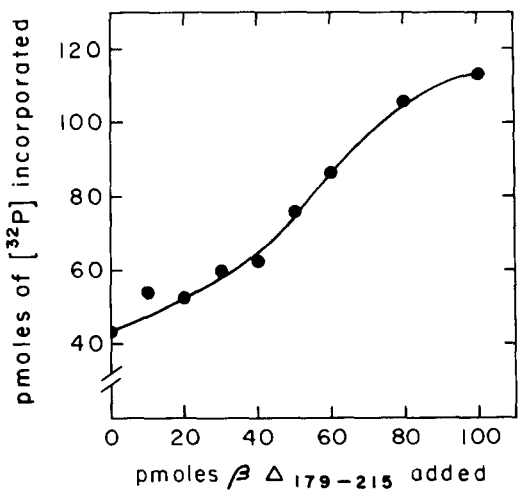

Fig. 2. The effect of high concentrations of $\beta_{\Delta 179-215}$ on CK2α phosphorylation of casein. The assay of CK2 phosphorylating activity was carried out as described in Methods using 2 pmoles of purified recombinant CK2α.

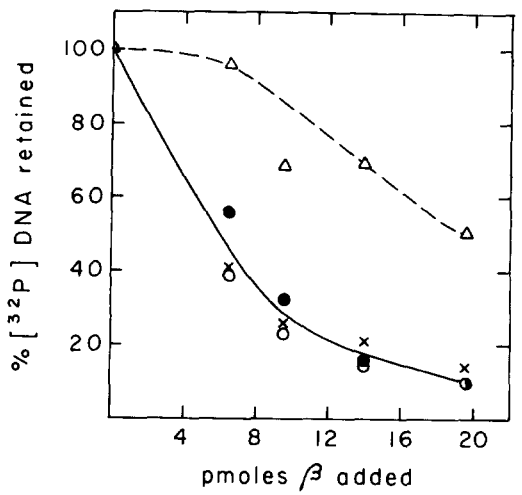

Fig. 3. The effect of β concentration on the binding of [^{32}P]DNA by CK2α. The assay for the binding of radioactive DNA to CK2α used the retention of the complex on nitrocellulose membranes as described in Methods. 7 pmoles of CK2α were used. In the absence of added β, approximately 30,000 cpm of [^{32}P]DNA was retained on the filters. This amount of radioactivity was set as 100% retention. ●, β_{WT}; ×, $\beta_{P\rightarrow A}$; ○, $\beta_{DEE\rightarrow AAA}$; △, $\beta_{\Delta 179-215}$.

The results obtained with the $\beta_{HHH\rightarrow AAA}$ mutant indicate that this unusual histidine cluster does not seem to play a very essential role in the regulation of the catalytic activity of the holoenzyme.

The use of different assays to test the activity of β mutants is useful because it provides other parameters to test the various effects of the regulatory subunit on CK2α properties. We have included the assay that involves the decrease in the interaction of CK2α with DNA caused by β [6]. This assay gives different results from the measurement of the phosphorylating activity since the $\beta_{DEE\rightarrow AAA}$ and $\beta_{P\rightarrow A}$ mutants do not show any hyperactivity with respect to β_{WT}. This observation means that the down-regulation of the phosphorylation activity caused by the mutated region of β involves a more subtle effect that can be observed in the rapidly turning over reactions involved in catalysis, but not in the stoichiometric interactions determined by the DNA binding assay. It is intriguing, however, that this DNA binding assay seems to be more sensitive to detect interaction of $\beta_{\Delta 179-215}$ with CK2α than the phosphorylation assay or the direct measurement carried out by sucrose gradient sedimentation since a very significant effect is observed at ratio of $\beta_{\Delta 179-215}$ to α of 2:1 and 3:1 in which very little effect is observed with the kinase assay. One explanation may be that DNA may increase the affinity of CK2α for some of the regions of β remaining in the $\beta_{\Delta 179-215}$ mutant.

The results observed with the effect of the mutants on the efficiency of inhibition by copoly(Glu:Tyr) (4:1) and on the stabilization of CK2α to thermal inactivation corroborate the findings that indicate a lower interaction of the $\beta_{\Delta 179-215}$ mutant.

Further mutational and structural studies are necessary for a more comprehensive understanding of the structural features of subunit β that are responsible for the up-regulation of CK2α activity. In addition, the physiological factors that may regulate the availability and the effects of β on the holoenzyme also remain to be determined.

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