Structure of protein kinase CK2: dimerization of the human β-subunit

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Abstract Protein kinase CK2 has been shown to be elevated in all so far investigated solid tumors and its catalytic subunit has been shown to serve as an oncogene product. CK2 is a heterotetrameric serine-threonine kinase composed of two catalytic (α and/or α′) and two regulatory β-subunits. Using the two-hybrid system we could show that the α- or α′-subunits of CK2 can interact with the β-subunits of CK2, but not with other α- or α′-subunits. By comparison, the β-subunit of CK2 can interact with another β-subunit. Important amino acids for successful dimerization of the β-subunit were localized between amino acid residues 156 and 165. Furthermore, we identified residues between amino acid 170 and 180 which antagonize the dimerization.

Key words: Protein kinase CK2; Structure; β-Subunit; Dimerization

1. Introduction

Protein kinase CK2 is an ubiquitous enzyme found in all so far investigated eukaryotic organisms. The high degree of amino acid homology from yeast to man suggests an important role in the metabolism (for reviews, see [1–5]). Genetic studies in *Saccharomyces cerevisiae* [6,7] show that the catalytic subunit is essential for viability. Similar results were obtained using *Schizosaccharomyces pombe* [8]. As in *S. cerevisiae*, overexpression of the catalytic subunit did not lead to detectable phenotypic changes. By contrast, CK2β overexpression inhibits cell growth and cytokinesis, with formation of multiseptated cells. Whereas the function of the α-subunit can be clearly assigned to be catalytic and hence can be directly compared with other members of the protein kinase family [9], the physiological role of the β-subunit only slowly begins to emerge. With the availability of recombinant human CK2 [10] it was possible to assign it at least three basic properties: (i) modulating activity [10,11], (ii) stabilizing the structure [12] and (iii) determining substrate specificity at [11,13]. For some substates, e.g. calmodulin, the β-subunit is a negative regulator. Especially, the latter role will be possibly of greater importance in the future than can be currently assessed, since so far no regulatory mechanism has been found for protein kinase CK2. Whereas the cAMP-dependent protein kinase, which also consists of two catalytic and two regulatory subunits, can be activated by dissociation in the presence of cAMP, a similar mechanism has not been found for CK2. By contrast, the tetrameric CK2 holoenzyme can only be dissociated by boiling in SDS followed by PAGE analysis. Hence, it is unlikely that dissociation will ever occur in vivo once the tetrameric complex has been formed. Regulation of CK2 activity to wards different substrates probably involves modulators influencing the downregulatory capability of CK2α-subunits towards certain substrates [14]. In any case, knowledge of the structure how the tetrameric complex is formed will be a prerequisite in facilitating the understanding the regulatory mechanism of CK2α.

Initial biochemical and biophysical characterization of the recombinant human CK2 subunits revealed that the CK2 subunit behaved as a protein of the predicted molecular mass calculated from the cDNA sequence, i.e. 44,000 Da [3]. By contrast, the CK2β-subunit (26,000 Da) showed a Stokes' radius similar to that found for the catalytic subunit in the presence of 500 mM KCl but also under denaturing conditions (6 M urea, 2 M NaCl). One explanation was to assume a rod-shaped geometry for the CK2β-subunit, alternatively it was suggested to exist as a dimer. Sucrose density gradient analysis showed that the β-subunit moved to the same position as the α-subunit suggesting dimer formation, again a rod-shaped geometry was not be ruled out [15]. Hence, a genetic approach using the two-hybrid system was chosen [16]. In addition, several β deletion mutants from the carboxy-terminal end and three-point mutations were examined. This allowed mapping of the minimum amino acid sequence required for dimerization and identification of residues which attenuate dimerizations. Results obtained by the two-hybrid system suggest that at least one crucial sequence needed for dimerization is localized in the carboxy-terminal half between amino acid residues 156–165. However, the reason for the stable dimer formation is so far obscure but it may be required for the subsequent formation of the very stable holoenzyme complex.

While these experiments were just terminated a paper appeared which reported on the α-, α′- and β-subunit interaction of protein kinase CK2 [17] albeit with no specific foocus on the β–β dimerization.

2. Materials and methods

2.1. Materials

Plasmids encoding the GAL4 DNA-binding domain (pGBT9) or the GAL4 transcriptional activation domain (pGAD424), the sequencing primers for the two vectors and the yeast strain SFY526 [18] were obtained from Clontech. Oligonucleotides were ordered from Eurogentech (Belgium). The T7 sequencing kit was purchased from Pharmacia.

2.2. Plasmid constructs

For cloning of the cDNAs of human CK2α and β into the p-GAD424 and pGBT9 vectors, clones pBB3 (CK2α) and pBB4 (CK2β) [10] were digested with restriction enzymes *Nde*I and *Hin*dIII, the cDNA fragments were isolated from agarose gels, filled in and cloned into the *Sma*I site of the vectors. Correct insertion was confirmed by sequencing. The encoded fusion proteins contained the complete sequence of the human CK2α or β. For cloning of the carboxy-terminal CK2β deletions, the plasmid pBB4 was used as a template for polymerase chain reactions. The first primer contained the 20 first nucleotides of the coding region of the CK2β cDNA. The second primers were complementary to the

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20 nucleotides within the CK2β cDNA preceeding the deletions. At its 5′ end they contained additional nucleotides representing a stop codon and a *Bam*HI recognition site. The PCR products were digested with *Xba*I and *Bam*HI and cloned into the *Xba*I/*Bam*HI site of the pGAD424/CK2β construct. The entire sequences of amplified fragments were confirmed. For cloning of point mutations, already created mutants [15] served as PCR templates. The primers for the PCR contained the 20 first nucleotides and the 20 complementary last nucleotides of the coding region of the human CK2β cDNA and in addition a *Xba*I and *Bam*HI restrictiction site, respectively. Cloning procedure was as described for the carboxy-terminal deletion mutants. The plasmid pGAD-GH/CK2α′ encoding a fusion protein between the GAL4 activation domain and the entire human CK2α′ cDNA was isolated by screening a HeLa cDNA library in the vector pGAD-GH with the pGBT9/CK2β construct. Details of the screening procedure are described elsewhere (manuscript in prep.), but we essentially followed the protocol recommended by Clontech.

All plasmids were routinely maintained in *E. coli* DH5α.

2.3. Transformation and maintenance of yeast

Yeast strains were grown and manipulated according to the Clontech protocol. For two-hybrid experiments, the yeast strain SFY526 was transformed with 100 ng of various pGAD424 and pGBT9 plasmids. After selection of transformants single colonies were picked with a toothpick, transferred in parallel (i) to a fresh plate and regrown and (ii) to a Whatman No. 5 filter paper, which was further incubated on a fresh plate for 3 days. The filter was frozen for ~5 s in liquid nitrogen, then layered over a second filter, prewetted with Z-buffer (16.1 g/l $Na_2HPO_4 \times 7\ H_2O$, 5.5 g/l $NaH_2PO_4 \times H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4 \times 7\ H_2O$) which contained 0.27 ml 2-mercaptoethanol and 1.67 ml X-gal stock solution (20 mg/ml) per 100 ml. Incubation was at 30°C for up to 4 h or until colonies turned blue.

2.4. Measurement of β-galactosidase activity

Assays for β-galactosidase activity were performed essentially according to the Clontech protocol. Material from the plate which was prepared in parallel to the filter was used for inoculation of 5 ml cultures with synthetic medium. The cultures were grown until $A_{600} = 1$ was reached. 500 μl and 1 ml of each culture was transferred to a microcentrifuge tube and centrifuged for 5 s. The yeast cell pellet was resuspended in 100 μl synthetic medium, 700 μl Z-buffer with 2-mercaptoethanol, 50 μl 0.1% SDS and 50 μl chloroform were added. The mixture was vortexed for 30 s. The reaction was started by adding 160 μl of 4 mg/ml ONPG in 0.1 M phosphate buffer pH 7.0 and incubated for 1 h at 30°C. Reactions were terminated by the addition of 400 μl 1 M Na_2CO_3. After centrifugation for 10 min the A_{420} was measured. β-galactosidase activities were calculated in Miller units according to the following formula: units = $1000 \times A_{420}$/(culture volume in ml × incubation time in min × A_{600}).

Table 1
Quantitative determination of dimerization in the two-hybrid system of $CK2\beta^{wt}$ fused to GAL4-binding domain with C-terminal deletions and selected point mutations of CK2β fused to GAL4 activation domain

CK2 deletions fused to GAL4 activation domain	$CK2^{wt}$ fused to GAL4 binding domain	β-galactosidase activity Miller units
β (1–215^{wt})	β^{wt} (1–215)	7.5 ± 0.89[a]
β (1–200)	β^{wt} (1–215)	10.1 ± 1.89
β (1–193)	β^{wt} (1–215)	7.0 ± 1.51
β (1–180)	β^{wt} (1–215)	8.3 ± 2.83
β (1–170)	β^{wt} (1–215)	12.2 ± 5.19
β (1–165)	β^{wt} (1–215)	24.2 ± 11.17
β (1–155)	β^{wt} (1–215)	1.2 ± 1.67
β (1–149)	β^{wt} (1–215)	0.5 ± 0.48
$\beta^{A177,178}$	β^{wt} (1–215)	15.0 ± 0.83[b]
β^{A175}	β^{wt} (1–215)	13.0 ± 0.70
β^{A171}	β^{wt} (1–215)	10.1 ± 0.44

[a]Activity values are the average (± S.D.) of at least three independent sets of experiments.
[b]In the case of the single mutations two independent sets of experiments were carried out.

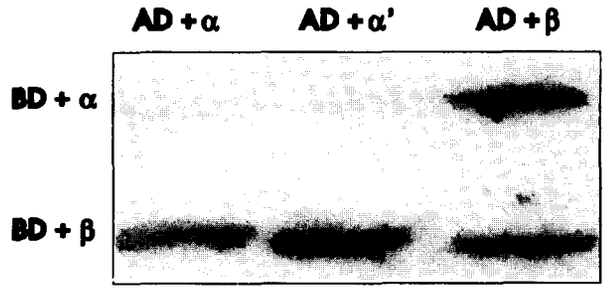

Fig. 1. Filter assay for β-galactosidase in order to test for qualitative interaction between the wildtype subunits of CK2 in the two-hybrid system. A streak of individual colonies harboring the CK2 subunit/GAL4 activation domain (AD) and binding domain (BD) constructs were allowed to grow on filters and then assayed for β-galactosidase activity. Positive interaction was visualized by the appearance of colored signals. Constructs used were: AD-α, AD-α′, AD-β, BD-α and BD-β.

3. Results and discussion

3.1. Dimerization of recombinant human CK2β

With the availability of recombinant human CK2 subunits [10] it was possible to carry out extensive biochemical and biophysical investigations. Sucrose density gradient analysis of the recombinant subunits revealed that the β-subunit (M_r 24.941 kDa) migrated to a similar position as the larger CK2α-subunit (M_r 45.141 kDa). These data support the notion that the β-subunit may exist as a dimer which is formed immediately after the expression of the recombinant human protein in *E. coli*. To prove this hypothesis, in vivo experiments were designed using the two-hybrid system [16]. The activation domain (AD) of the GAL4 cDNA was fused to the α, α′ or β cDNA and the binding domain (BD) to the α or β cDNA. Fig. 1 shows the results of such an experiment where different possible combinations of CK2 subunits were tested.

Cotransformation of the yeast strain SFY526 with different α, α′ and β hybrids were performed. Interaction between two-hybrid proteins were tested by the β-galactosidase filter assay. The obtained results clearly show an interaction between α/β, α′/β and β/β. There was no interaction between α/α and α/α′. β-Galactosidase expression was not detectable in yeast transformed with only one CK2 subunit hybrid alone or in combination with either the binding or activation domain of GAL4 (not shown).

3.2. Amino acid domain involved in the dimerization

Next we wanted to answer the question what is the minimum required protein size to allow β–β interaction. For this purpose, we created a set of deletion mutants from the carboxy-terminal end (Fig. 2), e.g. 1–200, 1–193, 1–180, 1–170, 1–165, 1–155 and 1–149. These mutants were fused to the activation domain of the GAL4 cDNA. Except for mutants 1–155 and 1–149, all mutants showed interaction with the $CK2\beta^{wt}$, suggesting that the crucial minimum protein size is between 1–156 (M_r ~17,000) and 1–165 (M_r ~18,000), comprising the sequence ^{156}GAYFGTGFPH^{165}. Therefore, one can assume a molecule size needed for β dimerization which is smaller than the minimum size needed for the subsequent interaction with the α-subunit which is between 1–180 and 1–193 [15].

The quantitative determination of β-galactosidase activity

involving the various mutants showed that there are significant differences among the mutants (Table 1). The wildtype and the deletion mutants (1–200; 1–193; 1–180; 1–170) showed activities between 7.1 and 10 U whereas mutants 1–155 and 1–149 are virtually lacking any significant β-galactosidase activity. The latter result is in agreement with the data we have obtained in the filter assay reaction (Fig. 2).

3.3. Attenuation of dimerization

Remarkably, however, is the 2 to 3-fold β-galactosidase activity increase observed with mutant 1–165 (24.2 U) in comparison to β^{wt} (7.5 U) and the deletion mutants 1–200; 1–193; and 1–180 (Table 1). Since an increase in β-galactosidase activity directly reflects an increase in dimerization intensity, we assume that the removal of amino acid residues 166–215 involves a loss of amino acid residues antagonizing dimerization as can be seen by a lower β-galactosidase activity (Table 1). Also mutant β1–170 (12.2 U) shows still a slightly elevated β-galactosidase activity whereas in mutant β1–180 wildtype activity was measured.

This suggested to us that somewhere between amino acid residues 170 and 180 might be a stretch of amino acids which are responsible for attenuation of dimerization. Tackle this problem, we created a set of exchange mutants, e.g. $A^{177,178}$ (Lys, Arg→Ala), A^{175} (Arg→Ala) and A^{171} (His→Ala). We focused on the sequence motif ^{175}RPKRP^{178}, where two basic amino acids are flanked by prolines and which is 100% conserved in the CK2 subunit from man, bovine, *Drosophila melanogaster* and *Xenopus laevis*. It is still 93% conserved in nematodes. The mutants A^{175} and $A^{177,178}$ which were already studied earlier [15] behaved in most experiments like CK2β^{wt}. Only in western blots with the purified proteins a strong band of the

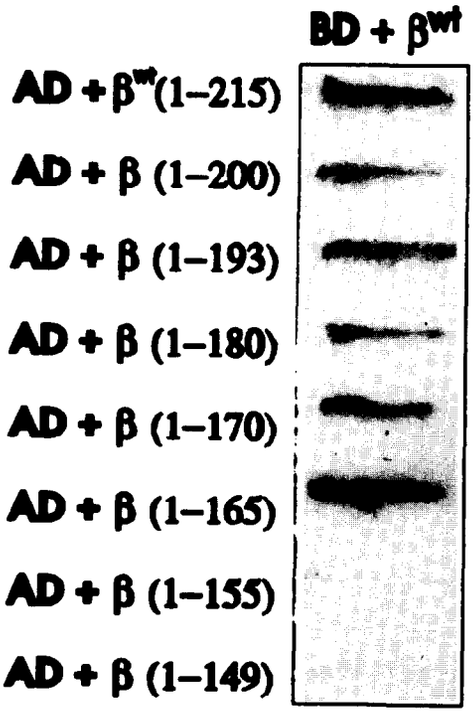

Fig. 2. Filter assay for β-galactosidase to test for qualitative interaction between the BD-β^{wt} and various AD constructs containing β^{wt} but also different deletion mutants. For further details, refer to the legend in fig. 1.

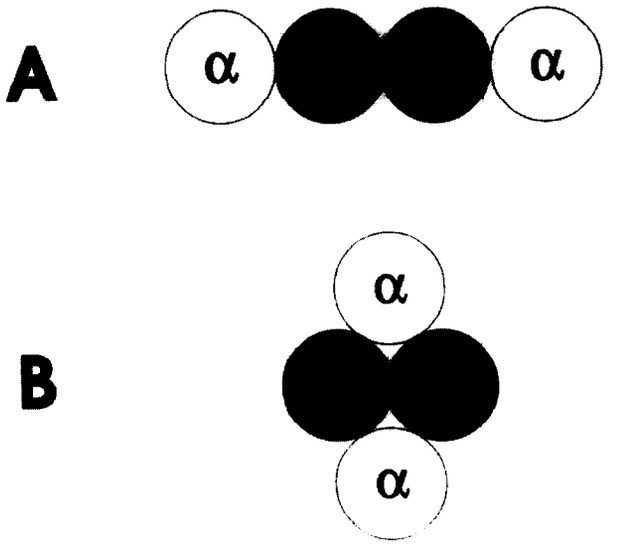

Fig. 3. Hypothetical model of human CK2 tetramer arrangement basing on the observation that recombinant human β-subunit behaves like a dimer according to its Stokes' radius and with respect to the results in the two-hybrid system. As a first step, the formation of β–β homodimers is postulated followed by the association of the α- and/or α′-subunits.

molecular mass of a CK2β dimer appeared (not shown). This led us to the assumption that the amino acids 175, 177 and 178 might play a role in dimerization. Indeed, Table 1 shows, that the double mutant $A^{177,178}$ where two basic residues were changed for alanines, has a 2-fold higher β-galactosidase activity than the wildtype. In case of the A^{175} mutant, where only one arginine of the ^{175}RPKRP^{178} sequence motif has been changed β-galactosidase activity is al ready lower than in the case of the double mutant and it is like the wildtype in the case of mutant A171 where a histidine, which is not part of the ^{175}RPKRP^{178} motif, was changed to alanine for control purposes.

Many biologically important peptide sequences contain proline. The conformational restrictions imposed by proline motifs in a peptide chain appear to imply important structural or biological functions as can be deduced from their often remarkably high degree of conservation found in many peptides and proteins [18] as is also the case for CK2β, too. The conformational constraints induced by proline residues in a peptide usually restrict proteolysis. Furthermore, the presence of a set of basic amino acids surrounded by prolines may be supportive in antagonizing otherwise stable structural domains. This could explain that deletion of this domain leads to a more intimate association of two molecules than it is commonly found between two wildtype molecules. From all these information, it is obvious that preferably mutations of the basic residues would possibly lead to a closer insight in their role in the dimerization capability of the β-subunit. Mutations affecting the prolines are prohibitive since they may lead to so drastic changes on the overall structure.

Gietz et al. [17] found the strongest interaction for the α–β and α′–β interaction but no interaction between α–α. In an earlier paper [19], it was shown that the majority of newly synthesized β-subunit is rapidly incorporated into complexes with the β-subunit. By comparison, the β-subunit is synthesized

in excess of α and incorporates slowly with α. All these data, together with recent findings, where β–β interaction was also shown by chemical crosslinking (W. Pyerin, pers. commun.) and biophysical studies using analytical ultracentrifugation (Flossdorf et al. in prep.) support the notion that β dimer formation precedes incorporation of α or α′-subunits. Fig. 3 shows two cartoons of the hypothetical holoenzyme structure. They differ from previous ones [20,21] inasmuch that they are based on the observation that the β–β dimerization is a prerequisite for the subsequent association of the α-subunits to complete the $\alpha_2\beta_2$ structure.

Clearly, we have to await the complete structural analysis of CK2 subunits by X-ray crystallography to obtain the final answer for the arrangement of the individual subunits in the tetrameric complex.

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