A-Raf kinase is a new interacting partner of protein kinase CK2 β subunit

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Abstract In a search for protein kinase CK2 β subunit binding proteins using the two-hybrid system, more than 1000 positive clones were isolated. Beside clones for the α′ and β subunit of CK2, there were clones coding for a so far unknown protein, whose partial cDNA sequence was already deposited in the EMBL database under the accession numbers R08806 and Z17360, for the ribosomal protein L5 and for A-Raf kinase. All isolated clones except the one for CK2 β showed no interaction with the catalytic α subunit of CK2. A-Raf kinase is a new interesting partner of CK2 β. The isolated A-Raf clone represented amino acids 268–606, but also a full length A-Raf clone interacted with CK2 β. At the site of CK2 β, residue 175 and amino acids between residues 194 and 200 are likely to be involved in direct interaction.

Key words: Protein kinase CK2; CK2 β subunit; A-Raf kinase; L5; Interaction; Two-hybrid system

1. Introduction

Protein kinase CK2 is a ubiquitously expressed and highly conserved pleiotropic serine/threonine protein kinase (for reviews see [1–3]). Its activity and quantity have been shown to be elevated in proliferating cells. Despite numerous reports on its potential role in proliferation and tumorigenesis, the regulation and physiological role of CK2 are not understood. CK2 consists of two catalytic (α) and two regulatory (β) subunits, which form the tetrameric $\alpha_2\beta_2$ holoenzyme. Once formed, the holoenzyme cannot be dissociated into its subunits except under denaturing conditions. The role of the β subunit in the holoenzyme complex is to modulate the activity of the α subunit, to contribute to the substrate specificity and to confer stability to the α subunit [4,5]. Furthermore the stimulation of CK2 activity by polybasic compounds is mediated through the β subunit [6]. In two-hybrid experiments it has been shown that one β subunit can interact with another β subunit, but also with the α or α′ subunit [7,8]. This supports the notion that β dimerization precedes binding of two α subunits and formation of the holoenzyme. Moreover CK2 β also binds to other proteins, e.g. p53 and ribosomal protein L5 [9–11]. p53 copurifies with CK2 and its binding is mediated by the β subunit. However, there is some evidence that the β subunit might have some other roles in the cell beside as a subunit of CK2. Studies in *Schizosaccharomyces pombe* have shown that the overexpression of CK2 β leads to inhibition of cell growth and cytokinesis with formation of multiseptate cells, whereas overexpression of CK2 α did not lead to any visible alteration in the phenotype [12]. In exponentially growing tissue culture cells the β subunit is synthesized in excess of the α subunit, although a substantial fraction of the newly synthesized protein is rapidly degraded [13]. Moreover, an asymmetric expression of CK2 subunits was observed in human kidney tumors, i.e. there is an excess of the β subunit in tumors versus normal tissues when compared to CK2 α [14]. In order to answer the question whether CK2 β interacts with other proteins, a two-hybrid library was screened with CK2 β as bait. Among the positive clones A-Raf kinase and ribosomal protein L5 were found. A-Raf kinase is a new interacting partner, not described before. It is one of the three isoforms of Raf kinases in mammalian organisms. All are involved in the MAP kinase cascade [15]. Most is known about the c-Raf-1 isoform, which plays an essential role in the control of cellular proliferation and transformation. But little is known about potential specific functions and regulation of the two other isoforms A-Raf and B-Raf [16].

2. Materials and methods

2.1. Screening a two-hybrid library with CK2 β as bait

Human CK2 β was cloned into the vector pGBT9 [17] as a fusion with the Gal4 DNA binding domain [7] and transformed into yeast *Saccharomyces cerevisiae* strain HF7c [18]. Sequential co-transformation with a HeLa cDNA library with 7×10^6 independent clones cloned in pGADGH as a fusion with the Gal4 activation domain (Clontech) was carried out. Co-transformants were plated on synthetic medium lacking tryptophan, leucine and histidine and containing 20 mM aminotriazole. Growing clones were further monitored for activation of the LacZ reporter gene in filter and/or liquid assays (see below). From seven of the positive clones the library Gal4 DNA activation domain plasmid was isolated by growing them in synthetic medium lacking only leucine and sequenced.

2.2. Direct CK2 β/A-Raf interaction tests

The yeast strain HF7c was cotransformed with a full length Raf construct cloned in p533 and a CK2 β wild-type clone or CK2 β mutant constructs cloned in pGAD424 [7]. p533 is a derivative of pPC97 in which the Leu marker was replaced by the Trp marker of pPC86, in order to make selection possible for transformation together with pGAD424, containing the Leu marker. This full length A-Raf construct was kindly provided by U. Rapp (University of Würzburg, Germany). After selection of transformants a filter assay to monitor the expression of the LacZ reporter gene was carried out.

2.3. β-Galactosidase filter and liquid assay

For the filter assay single colonies were picked and transferred to a Whatman No. 5 filter paper which was further incubated on a fresh plate for 2–3 days. The filters were frozen in liquid nitrogen, then layered over a second filter prewetted with Z-buffer (16.1 g/l $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4{\cdot}7H_2O$) which contained 0.27 ml 2-mercaptoethanol and 1.67 ml X-gal (20 mg/ml in dimethylformamide) per 100 ml. Incubation was done at 30°C for up to 12 h.

For the liquid assay 5 ml cultures with synthetic medium were inoculated with single colonies and grown until $A_{600} = 1$. 500 μl and

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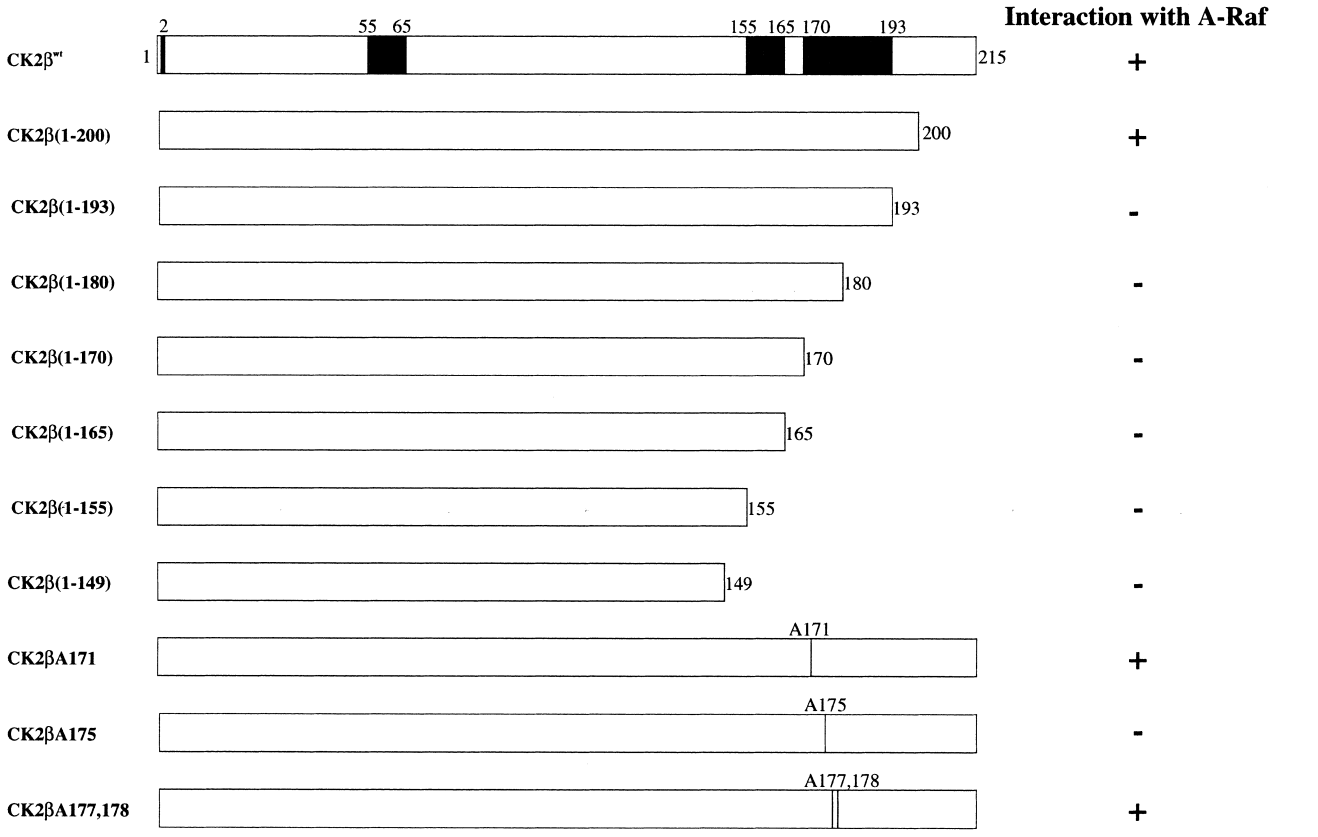

Fig. 1. Interaction of A-Raf with CK2 β^{wt} and mutants. In direct two-hybrid tests the interaction between the full length A-Raf and different CK2 β constructs was examined by activation of the HIS and LacZ reporter genes. +, both reporter genes were activated; −, neither of the reporter genes was activated. Numbers above refer to amino acids. Regions of CK2 β^{wt} with known functions are marked by boxes: 2, autophosphorylation site; 55–65, involved in α/β interaction and in polylysine effect; 155–165, critical for β/β dimerization; 170–193, involved in attenuation of dimerization and tetramer formation.

1 ml of each culture was transferred to a microcentrifuge tube and centrifuged for 5 s. The yeast pellet was dissolved in 100 μl Z buffer (see above) and frozen in liquid nitrogen. After thawing, 700 μl Z buffer with mercaptoethanol (see above) and 160 μl ONPG (4 mg/ml in Z buffer, freshly prepared) were added and the reaction was incubated for 1 h at 30°C. The reaction was stopped by addition of 400 μl 1 M Na_2CO_3, after centrifugation for 10 min in a microfuge at maximum speed, the A_{420} was measured. β-Galactosidase activity was calculated in Miller units according to the following formula: units=$1000 \times A_{420}$/(culture volume in ml × incubation time in min × A_{600}).

2.4. DNA sequencing analysis

The sequencing was carried out by the dideoxy chain termination method. Sequence homology searches were done using the GenEMBL database.

3. Results and discussion

3.1. Library screening

After transformation of the CK2 β construct containing cells with the library and plating, first approximately 3000 clones where obtained where the HIS reporter gene was activated. 164 of these clones appeared 3 days after plating, 1369 after 4 days, 813 after 5 days and 645 after 7 days. Of the first 164 clones all were able to activate the second reporter gene LacZ, whereas in only approximately half of the later appearing clones the second reporter gene was activated. Altogether we had isolated ~1500 clones positive for both reporter genes. However, only the first 164 clones represent probably strong interacting partners of CK2 β. From these first 164 clones seven were randomly chosen for further analysis. The containing library activation domain fusion construct was isolated and sequenced. In six cases sequence homology search identified the encoded proteins. We found the CK2 β subunit three times and the CK2 α′ subunit, the ribosomal protein L5 and the A-Raf kinase once. The sequence of the remaining clone #35 matched with more than 90% sequence identity two sequences in the database (accession numbers R08806 and Z17360), but the encoded protein is so far not known.

The CK2 β and α′ clones contained full length cDNAs, whereas both the L5 and the A-Raf clone contained only a 3′-part of the cDNA; in the case of L5 it started with the

Table 1
Detection of interaction between CK2 β and library proteins from the two-hybrid screen

Library protein	Growth on −His	β-Galactosidase	
		color	activity[a]
CK2 β	+	blue	3.1 ± 0.19
CK2 α′	+	blue	19.3 ± 2.33
#35	+	blue	1.1 ± 0.20
L5 (108–296)	+	blue	5.8 ± 0.19
A-Raf (268–606)	+	blue	3.5 ± 0.35

The interaction was determined by growth on −His medium (activation of HIS reporter gene) and quantitative and qualitative assays for β-galactosidase (activation of LacZ reporter gene).
[a]Activity values are given in Miller units as mean values ± standard deviation from three different experiments.

codon for amino acid 108, in the case of A-Raf with the codon for amino acid 268, both cDNA sequences ended with the poly-A tail.

The interaction between CK2 β and the 5 identified library constructs is shown in Table 1. The CK2 β/ CK2 α′ interaction seems to be the strongest (19.3 Miller units), whereas all others are in a lower range between 1.1 and 5.8 Miller units. In this latter range also interaction between CK2 β and α was determined (not shown). A similar difference in strength of interaction between CK2 β and the two CK2 α isoforms has been previously published by Gietz et al. [8].

All obtained clones were tested to see whether they would also interact with the catalytic CK2 α subunit. This was the case for none of the clones except CK2 β.

The identified interacting partners CK2 β and α′ did not come as a surprise, also interaction with ribosomal protein L5 was reported recently by Kim et al. [11]. In a two-hybrid screen with CK2 β they found the C-terminal portion of L5 starting with amino acid 153, whereas the clone isolated here started at amino acid 108. A new interacting partner for CK2 β is the A-Raf kinase.

3.2. Interaction of CK2 β with A-Raf

The isolated A-Raf clone interacting with CK2 β contained the region of the cDNA coding for amino acids 268–606. This region includes the kinase domain of A-Raf [19]. The interaction between CK2 β and A-Raf was further investigated using a full length A-Raf construct. This construct also revealed interaction with CK2 β. In contrast to a truncated molecule consisting of only the kinase domain the full length A-Raf is not catalytically active by itself and seems to have a different conformation [16]. However, it still allows interaction with CK2 β.

Several CK2 β C-terminal deletion mutants (1–200, 1–193, 1–180, 1–170, 1–165, 1–155 and 1–149) and three point mutations (A171, A175 and A177,178) were tested in order to map the specific sites required for that interaction. Direct two-hybrid tests were performed using the full length A-Raf and the different CK2 β deletion and mutation constructs (Fig. 1). The deletion mutant CK2 β (1–200) did reveal interaction with A-Raf, but the next deletion mutant (1–193) had lost this ability. Also all larger deletions were negative. Since CK2 β (1–193) is still capable of interacting with CK2 α (Boldyreff and Issinger, unpublished results) one should assume that this deletion causes no big structural changes. This suggests that residues 194–200 could be at least part of the binding site for A-Raf. The three point mutations of CK2 β tested for interaction with A-Raf led to a rather surprising result. One mutant, CK2 β A175, completely abrogated the interaction, whereas the others were able to interact. CK2 β residues 175, 177 and 178 seemed to be involved in attenuation of the β-β dimerization [7]. But in all biochemical tests the corresponding mutants behaved like wild-type β, especially when they were tested to replace the wild-type β in a tetrameric complex with CK2 α [20]. Therefore it seems unlikely that the single exchange mutant A175 leads to big conformational changes which might be the reason for prevention of A-Raf interaction. It seems more likely that residue 175 of CK2 β is directly involved in interaction.

Amino acids critical for interaction with the α subunit were mapped to amino acids 152–200 [21] and to 170–193 (Boldyreff and Issinger, unpublished results). Therefore it could well be that binding of A-Raf to CK2 β interferes with binding of CK2 α. Since CK2 β exists as a dimer that interacts with two CK2 α monomers to form the active, heterotetrameric CK2 enzyme, heterotetramerization with e.g. two A-Raf molecules might be also possible.

Our data suggest that CK2 β could be involved not only in forming a tetrameric protein kinase CK2 holoenzyme but also in binding to and possibly regulating other proteins. Both interacting proteins, ribosomal protein L5 and A-Raf kinase are interesting partners of CK2 β opening a new area for the understanding of the physiological role of protein kinase CK2 and its subunits.

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