Dissection of the dual function of the β-subunit of protein kinase CK2 ('casein kinase-2'): a synthetic peptide reproducing the carboxyl-terminal domain mimicks the positive but not the negative effects of the whole protein

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Abstract The dual function of the regulatory β-subunit of protein kinase CK2 is highlighted by its ability to abolish calmodulin phosphorylation in contrast to its stimulatory effect on the phosphorylation of peptide substrates. Here we show that a synthetic peptide reproducing the C-terminal region of the β-subunit (β[170–215]) stimulates to a similar extent the phosphorylation of either the peptide substrate or calmodulin and also protects the catalytic α-subunit against thermal inactivation as efficiently as full-length β-subunit. These data show that the positive and negative functions of the β-subunit reside in physically separated domains and that the elements responsible for positive regulation are located in the C-terminal region.

Key words: Protein kinase CK2; Casein kinase-2; Protein phosphorylation; Calmodulin phosphorylation; CK2 β-subunit

1. Introduction

Protein kinase CK2 (formerly termed casein kinase-2 [1]) is a ubiquitous and pleiotropic Ser/Thr protein kinase whose implication in a variety of cellular functions is supported by a number of observations [2–4]. CK2 phosphorylates a wide spectrum of target proteins, several of which are involved in gene expression and signal transduction, and its activity is invariably increased in transformed and proliferating cells. These observations would imply some kind of cellular control, which, however, is not evident in vitro, where CK2 is constitutively active toward most of its substrates. In searching for mechanism(s) capable of modulating CK2 activity, special attention has been devoted to its quaternary structure, composed of two catalytic (α and/or α') and two non-catalytic (β) subunits. The resulting holoenzyme is very stable and it does not dissociate except under denaturing conditions. Reconstitution experiments with recombinant subunits in conjunction with the use of the atypical substrate calmodulin, whose phosphorylation is prevented instead of being increased by the β-subunit [5], disclosed a dual function: on the one hand the β-subunit increases the stability and the overall catalytic activity toward canonical substrates, while on the other hand it imposes an intrinsic down-regulation. This latter is evident with calmodulin, while being masked by the positive effect with canonical substrates [6,7]. Mutagenesis experiments suggested that while the negative regulation and its consequent relief by polybasic effectors are mediated by acidic residues in the N-terminal moiety of the β-subunit, positive regulation is mainly exerted by the carboxyl-terminal domain [6]. In order to check the validity of this hypothesis and to dissect the positive and negative roles of the β-subunit we have started a project aimed at the chemical synthesis of large fragments of the β-subunit to test their ability to mimick the diverse functions of the whole protein. Here, we show that a synthetic peptide reproducing about one-fifth of the whole sequence, from residue 170 to the C-terminus, is able to protect against thermal inactivation and to stimulate up to 3-fold the basal activity of the catalytic subunit. However, it fails to suppress the phosphorylation of calmodulin; on the contrary calmodulin phosphorylation is stimulated in a mnaaer similar to that of canonical substrates.

2. Materials and methods

Synthetic peptides MSSSEEVSW, DLEPDEELEDNPNQSDL and NFKSPVKTIR, corresponding to the segments 1–9, 55–71 and 206–215 of the CK2 β-subunit, respectively, were prepared as previously described [8,9]. The peptide VHPEYRPKRPANQFVPRLYGFKIHPMAYQLQLQAASNFKSPVKTIR, reproducing the 170–215 segment of the CK2 β-subunit, and its shorter fragment 170–192, have been prepared using Fmoc chemistry on an automatic synthesizer (Model 431-A, Applied Biosystems) equipped with a UV-detector (ABI Model 759 A) for the monitoring of the Fmoc deprotection. The C-terminal amino acid was linked to a hydroxy-methylphenoxymethyl resin (Wang resin) (0.05 mmol of 0.96 mmol·g/resin). The following acylation step was carried out by Fmoc amino acids (10-fold excess) with appropriate side chain protecting groups, activated with *N,N*-dicycloexylcarbodiimide/1-hydroxybenzotriazole chemistry. After every coupling step the peptidyl resin was capped with acetic anhydride. Side chain protection was as follows: 2,2,5,7,8-pentamethylchroman-sulfonyl for arginine; trityl for asparagine, glutamine and histidine; *tert*-butyl for glutamic acid, serine, threonine and tyrosine; *tert*-butyloxycarbonyl for lysine. After the final N-terminal deprotection the peptide was cleaved from the resin with trifluoroacetic acid/anisole/ethanedithiol/ethyl methyl sulfide (95:3:1:1) and purified by weak cation-exchange chromatography. The purity (>95%) was evaluated by amino acid analysis and analytical high-pressure liquid chromatography. The synthesis of the peptide substrate RRRAADSDDDDD used in the present study was accomplished as previously described [10].

Rat liver CK2 was prepared essentially as previously described [11] with a further FPLC MonoQ chromatographic step. Its specific activity was 236 U/mg, 1 U being defined as the catalytic activity transferring 1 nmol of P per min into casein (80 μM) under standard conditions. Recombinant CK2α and -β subunits were prepared as previously described [12]. The specific activity of CK2α preparation used in this study was 52 U/mg.

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Phosphorylation conditions with either the synthetic peptide RRRAADSDDDDD (100 μM) or calmodulin (2.6 μM) as phosphorylatable substrate (final volume 50 μl) and evaluation of the phosphate incorporated were as previously described [7]. Whenever the activity of the reconstituted holoenzyme (α plus β subunits) was assayed in the presence of β-derived peptides, these were mixed together with the β-subunit prior to their addition to the α-subunit. Thermal stability was determined from the residual catalytic activity after preincubation of the samples at 45°C [6]. Sucrose gradient ultracentrifugation was performed as previously described [6].

3. Results

A schematic representation of the CK2 β-subunit with its putative functional domains and the corresponding synthetic peptides employed in this study are illustrated in Fig. 1A. The effect of these peptides on the activity of the α-subunit (tested on the peptide substrate RRRAADSDDDDD) is shown in Fig. 1B. While the peptide β[55–71] leads to a slight decrease in activity, the peptides β[170–215] and β[170–192] stimulate the kinase activity up to 2-fold, thus behaving like the intact β-subunit, though much less efficiently since stimulation by the whole β-subunit is several fold more pronounced (see below). In contrast shorter peptides reproducing the amino- and the carboxyl-terminal ends, β[1–9] and β[209–215], respectively, are ineffective up to 100 μM concentration.

The effect of peptides β[55–71], β[170–215] and β[170–192] on the catalytic activity of the α-subunit (see Fig. 1B) is not detectable if they are added to the reconstituted holoenzyme (not shown). This finding is consistent with the concept that the peptides influence CK2 activity by mimicking the effects of the corresponding regions of the β-subunit. Such a point of view is corroborated by the finding that the C-terminal peptide β[170–215] counteracts in a dose dependent manner the effective association of β- and α-subunits, as judged by activation experiments (Fig. 2). The ability of β[170–215] to compete out the β-subunit was also highlighted by parallel ultracentrifugation experiments, showing that the C-terminal peptide also prevents the reconstitution of normal $\alpha_2\beta_2$ heterotetramers (Fig. 2, inset).

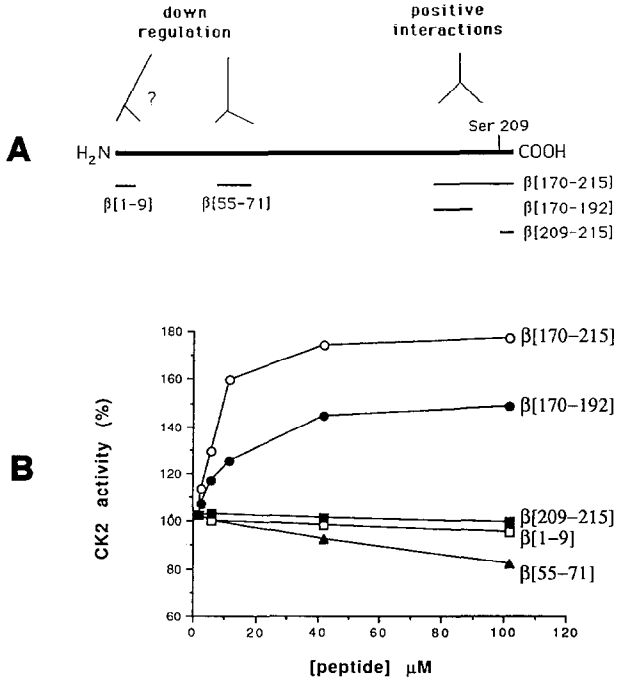

Fig. 1. Schematic representation of the β-subunit (A) and influence of β-subunit derived peptides on the catalytic activity of the α-subunit (B). The sequence of the β-subunit with its putative functional domains is schematically shown in A. Ser-209 represents the cdc2 kinase phosphorylation site. The activity of the α-subunit (0.05 μg) either alone or in the presence of increasing concentrations of β-derived peptides (as indicated) was evaluated using the peptide substrate RRRAADSDDDDD as previously described [7].

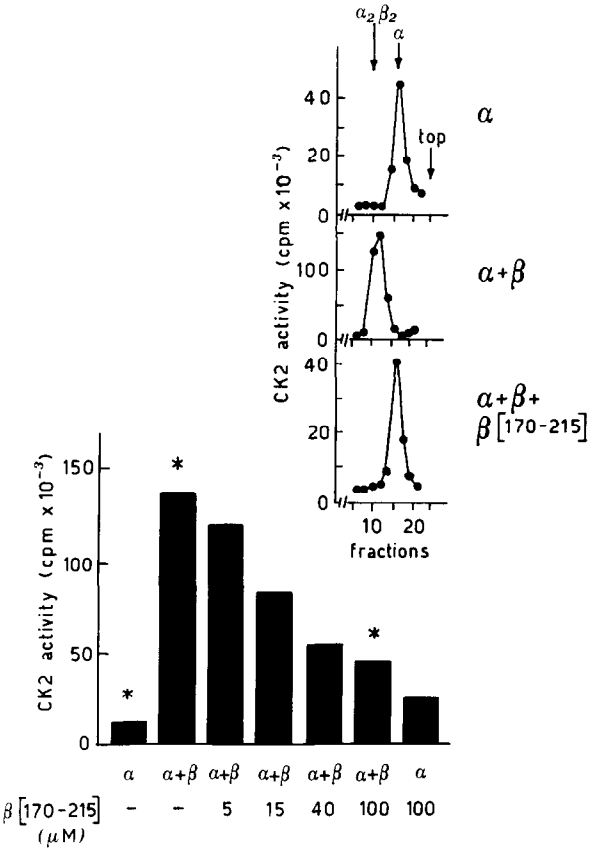

Fig. 2. Peptide β[170–215] counteracts the stimulation of CK2α activity by the β-subunit by preventing holoenzyme reconstitution. The α-subunit (0.13 μM) was incubated as in Fig. 1B in the presence and absence of equimolar amounts of the β-subunit and β[170–215] peptide as indicated. Aliquots of all the samples were assayed for catalytic activity using the peptide substrate RRRAADSDDDDD. Aliquots of the samples denoted by an asterisk were also analyzed for the presence of free α-subunit and reconstituted $\alpha_2\beta_2$ holoenzyme by sucrose density gradient ultracentrifugation (inset).

A third line of evidence that the C-terminal peptide β[170–215] possesses the elements responsible for positive interactions with the α-subunit is provided by the heat denaturation experiments of Fig. 3. Protection against thermal inactivation is one of the criteria for evaluating the effective binding of the β-subunit to the α-subunit, the latter alone being intrinsically very heat labile [9]. As shown in Fig. 3, a similar protection is provided by the β[170–215] peptide. Interestingly no significant protection could be observed with the truncated peptide β[170–192], despite its ability to stimulate CK2 activity almost as effectively as β[170–215] (see Fig. 1B).

In order to further strengthen the conclusion that the positive

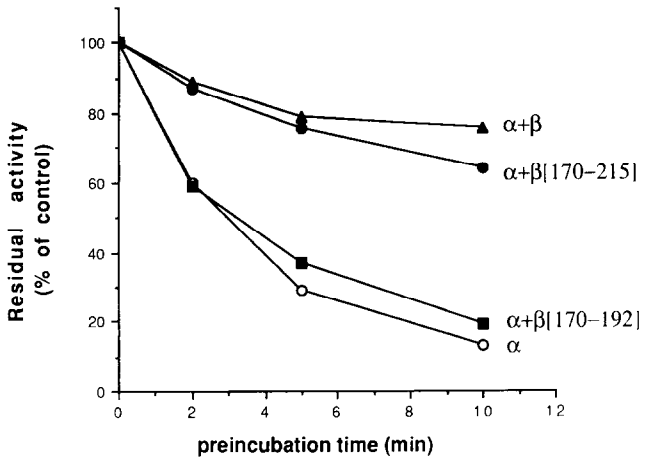

Fig. 3. Protection of α-subunit against thermal inactivation by the β-subunit and by the peptide β[170–215]. The catalytic activity of the α-subunit (0.05 μg), either alone or in the presence of equimolar amounts of β-subunit or 18 μM β-derived peptides, was evaluated after preincubation at 45°C for the time indicated.

effects of β[170–215] on both CK2 activity and stability are specifically due to its ability to mimick the properties of the C-terminal domain of the β-subunit, advantage has been taken of calmodulin, a CK2 substrate whose phosphorylation is prevented, rather than stimulated, by the β-subunit [5]. The acidic residues responsible for down-regulation of calmodulin phosphorylation are located in the N-terminal moiety of the β-subunit [7] and therefore are not included in the β[170–215] peptide. It was therefore expected that, unlike the β-subunit, this peptide would prove incapable of preventing calmodulin phosphorylation. Actually, as shown in Fig. 4 the peptide β[170–215] not only fails to suppress calmodulin phosphorylation but even stimulates it to the same extent observed with canonical peptide substrates. This provides clear-cut evidence that the positive and negative regulatory effects of the β-subunit can be physically dissected. It also supports the view that the β-subunit is a potential stimulator of CK2 activity also when calmodulin is the substrate, although this property is normally masked by the predominant negative influence of other parts of its molecule.

4. Discussion

Although the regulation of CK2 activity is still a matter of conjecture a breakthrough in this problem recently came from the observation that its non-catalytic β-subunit plays a dual role, by conferring improved stability and higher activity to the catalytic subunit while imposing, at the same time, a kind of intrinsic down-regulation on it [6,7]. The discrimination between these two opposite effects, whose balance ultimately determine the basal activity of CK2, was made possible by reconstitution experiments with mutated β-subunits [6,7] in conjunction with the use of substrates that perceive to different extents the 'positive' and 'negative' regulatory effects.

In particular a number of acidic residues clustered in the N-terminal moiety of the β-subunit (sequence 55–64 and possibly the autophosphorylation site at the very N-terminal end) were shown to be implicated in down-regulation [6,13], which becomes especially evident when calmodulin is the substrate [7]. In contrast the deletion of the carboxyl-terminal part, from residue 170 to the end, prevented the association with the α-subunit, and consequently protection against thermal inactivation and stimulation of basal activity [6]. We have now provided the tangible proof that indeed the carboxyl-terminal 170–215 domain exhibits positive regulation by mimicking both the protection against thermal inactivation and the stimulation of basal activity that are among the specific features of the β-subunit. Interestingly a truncated peptide (β[170–192]) lacking the last 23 amino acids is still able to stimulate basal activity but has lost the ability to prevent thermal inactivation. This would indicate that the stimulatory and protective potentials rely on distinct structural features, and that the latter but not the former requires the integrity of a segment downstream of residue 192. Interestingly the phosphoacceptor site for cdc2 kinase is located within this deleted segment (Ser-209), suggesting that its phosphorylation, which has been shown neither to affect activity [14] nor holoenzyme assembly [15], might be implicated in the stability of CK2 and hence its turnover.

By sharp contrast to the positive effects of the β-subunit, which are mimicked by the 170–215 peptide, the down-regulatory potential of the β-subunit is totally absent in this peptide, as proved by its inability to prevent calmodulin phosphorylation, which instead is stimulated to the same extent as that of canonical substrates, or even more so. This finding also suggests that calmodulin phosphorylation might not be exempt from the stimulatory effect of the β-subunit but that under basal conditions this effect is totally masked by overwhelming negative regulation, at least in vitro.

The lack of down-regulation by peptide β[170–215] is in agreement with the concept that this negative property is dependent on acidic residues in the N-terminal moiety of the β-subunit, with special reference to those in the 55–64 sequence [6,7]. This conclusion is further strengthened by the finding that a peptide reproducing the 55–71 sequence is in some way com-

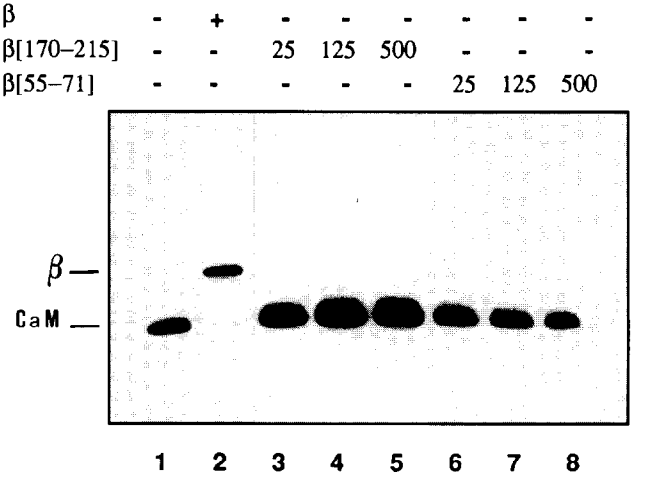

Fig. 4. Phosphorylation of calmodulin by CK2α subunit either alone (lane 1) or after addition of equimolar amounts of β-subunit (lane 2) and increasing amounts of peptides β[170–215] (lanes 3–5) or β[55–71] (lanes 5–7). The amounts of peptides β[170–215] and β[55–71] are expressed as their molar ratios vs. the α-subunit. Radiolabeled calmodulin was resolved by SDS-PAGE and detected by autoradiography [7]. The positions to where the β-subunit and calmodulin (CaM) migrated are indicated on the margin.

plementary to β[170–215] in that it fails to either protect or activate, while it significantly reduces the basal activity of the α-subunit, in a dose dependent manner.

It should be noted that both β[170–215] and, even more so, β[55–71] need to be in large molar excess over the α-subunit in order to display their effect, while the whole β-subunit is effective at equimolar concentrations. This does not invalidate the conclusion that our data reflect the mimetic properties of the peptides. The possibility in fact that their efficacy might be merely accounted for by unspecific interactions with the catalytic site of the enzyme is ruled out by two lines of evidence: firstly their effect is only evident with the α-subunit, but not, at comparable concentrations, with the holoenzyme composed of two α- and two β-subunits; secondly the β[170–215] peptide counteracts in a dose-dependent and reversible manner the stimulatory effect of the β-subunit by preventing its association with the α-subunit, as expected if they are competing for the same binding site(s).

It is not surprising on the other hand that the whole β-subunit would display much higher affinity for the α-subunit than peptides reproducing, to the best, 20% of its length (see Fig. 1A). The synthesis of larger β fragments and the generation of new truncated mutants will provide the tools for assessing whether high affinity binding is retained by the whole isolated activatory domain or strictly depends on the integrity of the β molecule inclusive of its down-regulatory site(s) as well.

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