Biochemical evidence that the N-terminal segments of the α subunit and the β subunit play interchangeable roles in the activation of protein kinase CK2

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Received 25 October 1998; received in revised form 8 November 1998

Abstract The concept that the amino-terminal segment plays a role in conferring high basal activity to protein kinase CK2 α subunit has been validated by generating two mutants (Y26F and Δ2–6) which are defective both in catalytic activity and in thermal stability. The additional finding that the activity of the two mutants is fully restored upon association with the regulatory β subunit, in conjunction with the observation that synthetic peptides reproducing the N-terminal segment (1–30) and the activation loop (175–201) of CK2α counteract the functional effects of the C-terminal domain of the β subunit, is consistent with a mechanism of activation of CK2 where the N-terminal domain of α and the C-terminal domain of β play interchangeable roles.

Key words: Protein kinase CK2; CK2 regulation; Casein kinase II; CK2 mutant; Protein phosphorylation

1. Introduction

Protein kinase CK2 (also known as casein kinase II) is a ubiquitous and pleiotropic Ser/Thr protein kinase which is highly conserved in evolution indicating a vital cellular role for this protein kinase [1,2]. CK2 phosphorylates seryl and threonyl residues which are specified by clusters of acidic determinants, the one at position *n*+3 defining the minimum consensus (S/T-x-x-E/D) [3,4].

More than 160 targets of CK2 are currently known, including enzymes involved in DNA, RNA and protein synthesis, transcription factors, signal transduction mediators and other nuclear factors [2]. CK2 activity is elevated in proliferating and transformed tissues, including leukemias and solid tumors [5–11]. A critical role of CK2 in the pathogenesis of cancer has been demonstrated by over-expressing its catalytic α subunit in lymphocytes of transgenic mice [12]. Moreover, the presence of CK2 α transgene collaborates with c-Myc and Tal-1 expression [12,13] and with p53 deficiency [14] in developing lymphomas in mice, while ectopic expression of CK2 α and, even more, α′, synergizes with Ha-Ras in inducing transformation of cultured fibroblasts [15].

These observations highlight the importance of deciphering the mode of regulation of CK2, which is still quite enigmatic. CK2 holoenzyme is composed of two catalytic (α and/or α′) and two 'regulatory' β subunits, to give very stable heterotetramers which apparently do not dissociate unless under denaturing conditions. There is evidence, however, that the free catalytic subunits could be present in the cell under special circumstances [15,16], and monomeric forms of CK2 composed of isolated catalytic subunits have been found in plants [17] and molds [18]. Both the holoenzymes and the isolated catalytic subunits are endowed with spontaneous basal activity tested with either casein or peptide substrates, and are not susceptible to tight control by either any known second messenger or phosphorylation. However, unlike the catalytic subunits displaying basal activity toward all substrates tested so far, the holoenzyme is inactive toward some protein substrates, notably calmodulin [2]. This led to the discovery of a dual function of the β subunit exerting both a positive and a negative control [19], whose balance may result either in stimulation of activity (as observed with most peptide substrates) or in inhibition, as typically observed with calmodulin [20]. Such opposite effects of the β subunit can be physically dissected by generating large peptides reproducing its N- (1–77) or C-terminal domains (155–215) mimicking either the inhibitory or the stimulatory effects of full length β, respectively [21]. In particular the C-terminal domain peptides are responsible for tight association to the catalytic subunits and invariably stimulate catalytic activity irrespective of the nature of the phosphoacceptor substrate, calmodulin included [21].

The recent elucidation of the crystal structure of *Zea mays* CK2α [22] has revealed a number of features that could account for its constitutive activity, the most remarkable being a tight contact between the N-terminal region and the 'activation loop' which is therefore displaced from the catalytic cleft and maintained in an open conformation. By contrast, in cyclin dependent protein kinases (CDKs), belonging to the same phylogenetic subgroup of CK2 [23], the N-terminal segment is lacking and the activation loop is oriented toward the opposite direction keeping the catalytic subunit in its fully inactive form. Partial activation of CDK2 requires the binding of cyclin which makes contacts, among others, with the activation loop, displacing it toward an open conformation [24] similar to the one which in CK2α is ensured by the N-terminal segment [22].

It is conceivable therefore that constitutive activity is conferred to CK2α and CK2 holoenzyme by two different structural elements, the N-terminal segment of α and the C-terminal domain of β, respectively, both operating through the activation loop. To validate this hypothesis we have generated

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Abbreviations: CDK, cyclin dependent protein kinase; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography

PII: S0014-5793(98)01516-6

two mutants of CK2α altered in their N-terminal segment (Y26F and Δ2–6), and we have synthesized large peptides reproducing the N-terminal segment and the activation loop, potentially able to antagonize functional interactions between the α and the β subunits. By these independent approaches we have obtained evidence that indeed the N-terminal segment of the α subunit and the C-terminal region of the β subunit play interchangeable roles in keeping CK2 in its active conformation.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. Recombinant calmodulin was kindly provided by Prof. E. Carafoli (Zürich, Switzerland). All other chemicals were obtained from Sigma. The two oligonucleotides used during the preparation of the αΔ2–6 mutant were synthesized by TIB MOLBIOL S.r.l.

2.2. Synthetic peptides

Peptide substrate RRRAADSDDDDD was synthesized as previously described [25]. The CK2α derived peptides were synthesized using an automated peptide synthesizer ABI 431-A (Applied Biosystems) equipped with HBTU/HOBt chemistry on a 4-(hydroxymethyl)-phenoxymethyl-copolystyrene-1% divinylbenzene resin (Perkin Elmer) and *N*-α-Fmoc amino acid derivatives (Novabiochem) according to our own protocols (for details see [21]).

The crude peptides were purified by HPLC on a preparative reverse column Prep Nova-Pak HR C18, 6 μm, 25×10 mm (Waters). The analytical HPLC and MALDI-TOF mass spectrometry analysis of the purified peptides showed a correct sequence and a purity of 95%.

2.3. Mutagenesis and expression of CK2 subunits

The αY26F mutant was generated by oligonucleotide directed mutagenesis as described in detail previously [26]. The N-terminal deleted mutant αΔ2–6 was obtained using the 'QuickChange site-directed' kit (Stratagene). The coding region of human α cDNA was inserted in vector pT7-7. Two synthetic oligonucleotide primers GGAGATATACATATGAGCAGGGCCAGAGTTTAC, each complementary to opposite strands of the vector, were used to delete 15 bp after the ATG starting codon. The obtained clones were sequenced and the positive clones were used to transform BL21(DE3) competent bacterial cells. Expression and purification of α wt, αY26F, αΔ2–6 and β subunit were performed as described by Grankowski et al. [27].

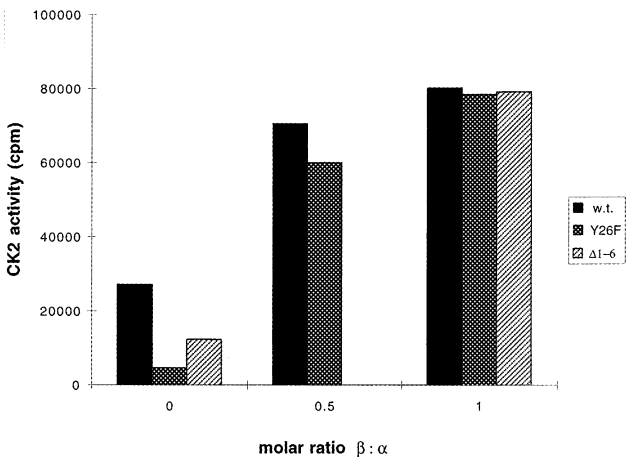

Fig. 1. Catalytic activity of CK2α wild type and mutants Y26F and Δ1–6 in the absence and presence of the β subunit. The activities of recombinant CK2 α wt, αY26F and αΔ1–6 (0.22 pmol) in the absence and presence of increasing amounts of the β subunit were assayed by using the specific peptide RRRAADSDDDDD (80 μM) as phosphorylatable substrate. Phosphorylation conditions and evaluation of ^{32}P incorporated are described in Section 2.

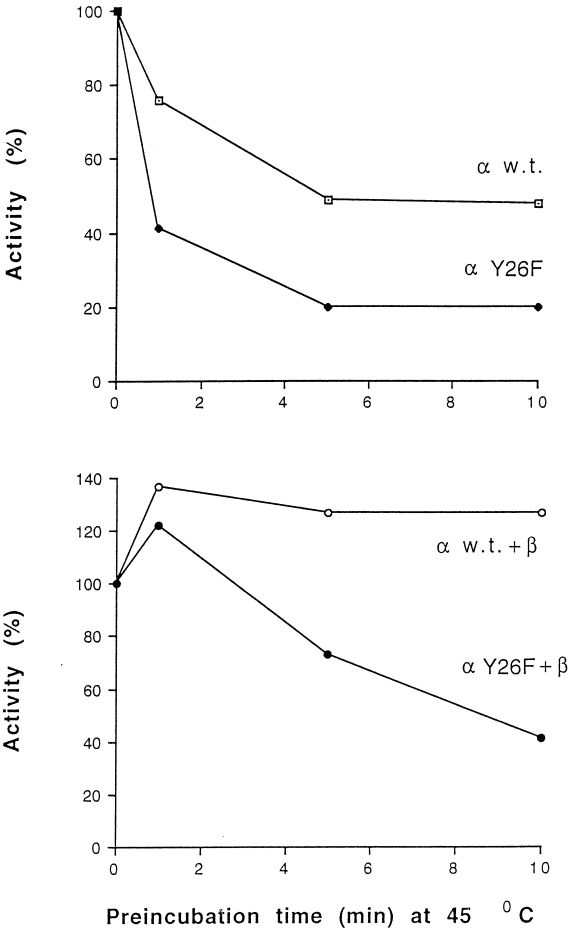

Fig. 2. Thermal stability of CK2α wild type and mutant Y26F either alone (A) or in the presence of equimolar amounts of β subunit (B). The effect of pre-incubation at 45°C for the times indicated of α subunit either alone (0.65 pmol) or in the presence of an equimolar amount of β subunit, was evaluated by assaying the residual activity, expressed as 100% of untreated controls, toward the peptide substrate RRRAADSDDDDD (80 μM). Phosphorylation conditions are described in Section 2.

2.4. Phosphorylation assay

Phosphorylation of synthetic peptide RRRAADSDDDDD (100 μM) was performed with 10 min incubation at 30°C in 30 μl of a medium containing 50 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 100 mM NaCl and 50 μM [γ-^{32}P]ATP (specific activity 300 cpm/pmol). The reaction started with the addition of the enzyme and ^{32}P incorporation was evaluated with the phosphocellulose paper procedure [28]. In the case of calmodulin phosphorylation and CK2 autophosphorylation experiments the reaction was blocked by the addition of Laemmli solution followed by SDS-PAGE and autoradiography as previously reported [21].

3. Results

Crystallographic analyses of mouse CK2α [22] have disclosed extensive contacts between the N-terminal region and the activation segment mediated by residues which are conserved in CK2 from different organisms. These interactions stabilize an open conformation of the activation loop which could account for the high basal activity of CK2 catalytic subunits as opposed to cyclin dependent protein kinase belonging to the same group of CK2, where this N-terminal segment is absent and interaction with cyclin is a prerequisite for activation.

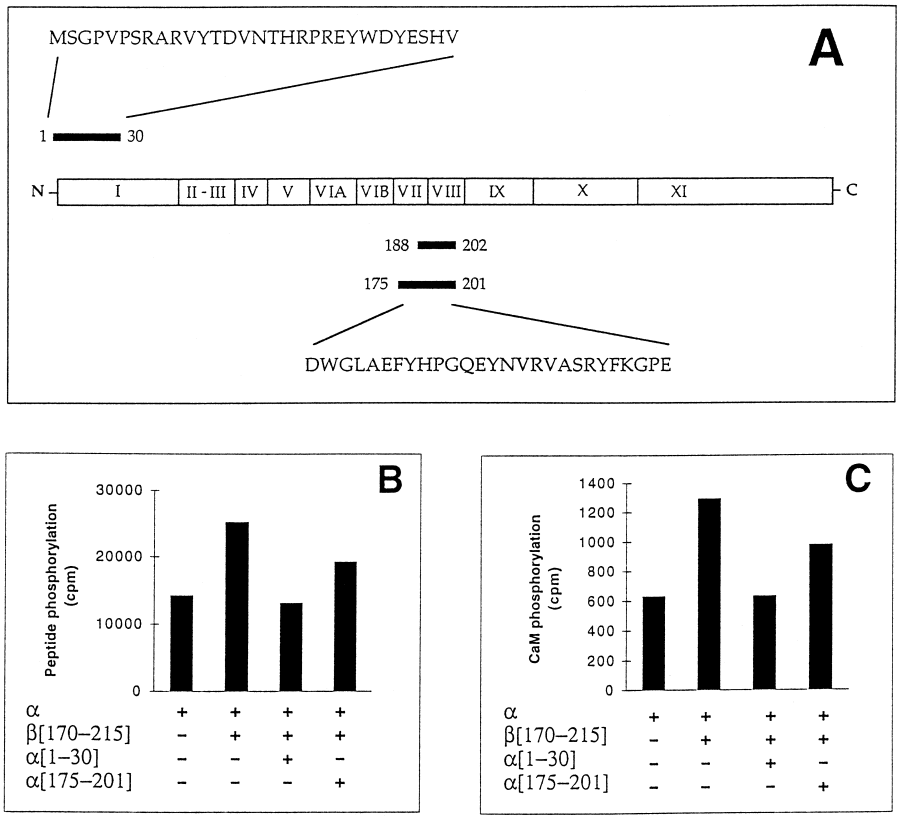

Fig. 3. The α[1–30] and α[175–201] synthetic fragments counteract the stimulation of catalytic activity by β[170–215]. The synthetic peptides α[1–30] and α[175–201], schematically illustrated in A, were added (80 μM) together with 40 μM β[170 -215] peptide in the phosphorylation assay of 80 μM RRRAADSDDDDD peptide substrate (B) and of 10 μM calmodulin (C) by CK2α subunit (2 pmol). Phosphate incorporation was evaluated and quantified as described in Section 2. Reported data are means of at least three independent experiments with S.E.M. not exceeding 15%.

To assess the actual relevance of these interactions in conferring high basal activity to CK2 one of the residues implicated, Tyr-26, whose side chain is connected by a hydrogen bond to Glu-180 in the activation loop of maize CK2, was mutated to Phe in human CK2α.

As shown in Fig. 1 the resulting mutant, Y26F, exhibits a markedly reduced catalytic activity toward a specific peptide substrate. This is accounted for by a drop in V_{max}, whereas the K_M values for either ATP or the peptide substrate are not appreciably affected (not shown). The Y26F mutant is still able to associate with equimolar amounts of the β subunit to give $\alpha_2\beta_2$ holoenzyme whose catalytic activity is indistinguishable from that of the wild type holoenzyme (Fig. 1). Consequently, as also highlighted in Fig. 1, the catalytic activity of Y26F is stimulated by the β subunit more than it is that of wild type (17.5-fold as compared to 2.9-fold, respectively).

In maize CK2α the most N-terminal residue that makes contact with the activation loop is Ser-2, which is the homolog of Ser-7 in human CK2α, where a short segment of five additional residues is present. This segment is also conserved in CK2α subunits from other vertebrates [2]. To check whether this additional N-terminal stretch is also implicated in stabilizing the active form of human CK2α, a deletion mutant, Δ2–6, was generated. Its catalytic activity, as shown in Fig. 1, is significantly reduced as compared to wild type, though not as much as that of Y26F. Like with mutant Y26F also the activity of the deletion mutant αΔ2–6 is fully restored to wild

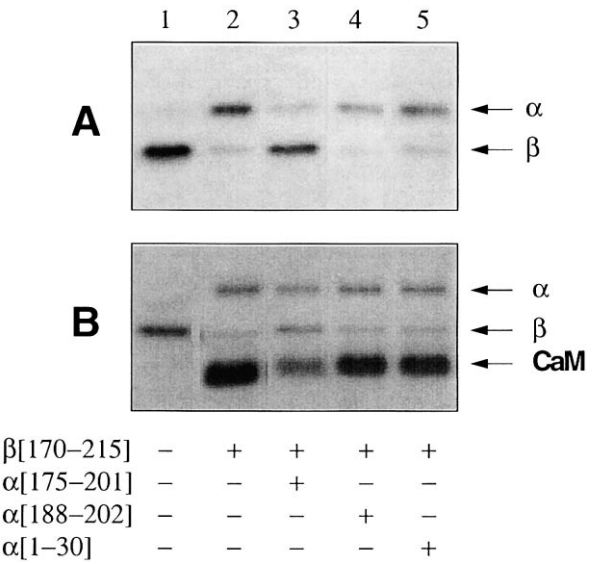

Fig. 4. Effect of the synthetic α peptides on CK2 holoenzyme autophosphorylation (A) and phosphorylation of calmodulin (B) in the presence of β[170–215]. Phosphorylation conditions are described in Section 2. Calmodulin was 10 μM. β[170–215] peptide (40 μM) and CK2α derived synthetic fragments (240 μM) were added as indicated. Recombinant CK2 holoenzyme (1.8 pmol) was purified from mixed extracts of α and β expressing bacteria as previously described [31]. Only the autoradiogram is shown.

type level upon addition of the β subunit (Fig. 1). The hyperstimulatory effect of the β subunit observed with mutants Y26F and Δ2–6 is also evident if the β subunit is replaced by its C-terminal peptide β[155–215] previously shown [21] to mimic positive regulation by β (not shown).

The Y26 to Phe mutation also promotes a decrease in thermal stability (Fig. 2A). Unlike catalytic activity, thermal stability is not fully restored to wild type level by association with the β subunit (Fig. 2B).

It has to be concluded from these experiments that alterations in the N-terminal segment cause significant drops both in catalytic activity and in thermal stability. While the former, however, is fully complemented by interactions with the β subunit, with special reference to its C-terminal domain, the latter persists also in the presence of the β subunit. These data support the view that upon the formation of the holoenzyme the β subunit replaces the N-terminal segment in keeping the activation loop in its open conformation.

In order to corroborate this concept two peptides reproducing the N-terminal segment (residues 1–30) and the activation loop (residues 175–201) were synthesized (Fig. 3A) and analyzed for their ability to antagonize some of the effects of a peptide reproducing the C-terminal region of the β subunit (residues 170–215).

As shown in Fig. 3B,C, the stimulatory effect of β[170–215], tested using either a specific peptide (B) or calmodulin (C) as phosphorylatable substrate, is abolished by the N-terminal α peptide (α[1–30]) and significantly reduced by the activation loop peptide (α[125–201]).

Peptide β[170–215], if added in large molar excess, interferes with the correct reconstitution of the $\alpha_2\beta_2$ tetramers [29] as judged by its ability to prevent inhibition of calmodulin phosphorylation [21] and to deeply alter CK2 holoenzyme autophosphorylation patterns [30]. While in fact CK2 holoenzyme normally autophosphorylates on its β subunits (residues Ser-2/3), in the presence of C-terminal β peptides, the autophosphorylation of the β subunit is suppressed and the autophosphorylation of α becomes evident [30]. As shown in Fig. 4A,B, both these effects are abolished if the peptide α[175–201] is added together with β[170–215]. A shorter peptide (α[188–202]), lacking the first part of the activation loop where the residues making the main contacts with the N-terminal segment are located [22], is nearly ineffective on both de-inhibition of calmodulin phosphorylation and inversion of the autophosphorylation pattern. This corroborates the view that the peptide α[175–201] displays its effect by competing against β-α interactions that are mediated by the activation loop, which, in the isolated α subunit, makes contact with the N-terminal segment.

Induction of calmodulin phosphorylation by β[170–215] was also counteracted by peptide α[1–30] (Fig. 4A), whose perturbing effect on the autophosphorylation pattern conversely was quite modest (Fig. 4B).

4. Discussion

The concept arising from crystallographic analysis of *Zea mays* CK2α that the N-terminal segment may play a crucial role in conferring high basal activity to the catalytic subunit by interacting with its activation loop [22] has been validated by showing that the mutation of Tyr-26 to Phe and the deletion of the N-terminal residues 2–5 in human CK2α cause a drop in catalytic efficiency. Tyr-26 is one of the three conserved residues mainly responsible for the contacts with the activation loop, and it was expectable that its mutation would have weakened these interactions. The 2–6 segment of human CK2α is conserved in CK2 from vertebrates, while it is not present in maize CK2α: our data show that it contributes with the rest of the N-terminal segment to confer to human CK2α its fully active conformation.

The additional finding that impaired catalytic activity of mutants Y26F and Δ2–6 can be entirely restored to wild type level by addition of equimolar amounts of the β subunit suggested that in CK2 holoenzyme the β subunit replaces the N-terminal segment in maintaining the activation loop in its open conformation. The exactness of this inference has been corroborated by showing that a number of functions of a peptide reproducing the C-terminal stimulatory domain of the β subunit, namely up-regulation of the catalytic subunit, de-inhibition of calmodulin phosphorylation by CK2 holoenzyme, alteration of autophosphorylation pattern, are counteracted to variable extents by peptides reproducing the activation loop (residues 175–201) and the N-terminal segment (residues 1–30).

The formation of the holoenzyme would therefore imply the displacement of the N-terminal segment from the catalytic core and its replacement by structural elements of the β subunit located in its C-terminal domain. In this respect the β subunit would operate like cyclin A in the active CDK2 complex where it keeps the activation loop in its 'open' conformation [24]. Pertinent to this could be the following observations: (i) the regions of the catalytic core of CK2α which make contact with the N-terminal segment (i.e. the 'Lys-rich stretch' and the 'activation loop') are homologous to sequences of CDK2 that interact with cyclin A [22]; (ii) despite the lack of overall similarity between the β subunit and cyclin sequences, some motifs of cyclin A that make contact with the catalytic subunit of CDK2 display sequence homology to segments of the β subunit: in particular four hydrophobic residues of cyclin A which interact with the PSTAIRE region of CDK2, namely F267, L299, F304 and L306, are conserved or conservatively replaced in the β subunit of CK2 (F159, I191, Y197 and L199) being located in that part of its C-terminal domain which is essential for physical interactions with the catalytic subunit [19,21]. It should be noted that the main counterpart of these residues in CDK2 is Ile-49, conserved in CK2α (Ile-78) where it makes a hydrophobic pocket with two residues of the N-terminal segment (V31 and W33) in maize CK2α crystallized without the β subunit.

In conclusion, a major functional difference between CK2 and CDKs would consist in the presence of an N-terminal segment which ensures basal activity to the isolated catalytic subunit of CK2, whereas it is absent in the catalytic subunits of CDKs which consequently are totally inactive. Another difference related to this consists in the role of the β subunits as compared to cyclins: while the latter are invariably activatory elements operating on otherwise inactive catalytic subunits, the β subunit displays a dual function by exerting both positive and negative regulation on a catalytic subunit which is already intrinsically active.

It is not clear what the fate of the N-terminal segment might be once it is displaced by the β subunit. While a definite answer to this question will require the solution of the crystal structure of complexes between α and β (or at least functional

β domains) it is likely that the N-terminal segment stabilizes the holoenzyme conformation by making new contacts within the tetramer. While in fact the reduced catalytic activity of mutant Y26F is fully restored upon holoenzyme formation, its reduced thermostability persists in the holoenzyme, consistent with the view that Tyr-26, once displaced from the activation loop, is engaged in new interactions relevant to thermostability.

Acknowledgements: This work was supported by grants from the Consiglio Nazionale delle Ricerche (97.03614.PS14 and T.P. on Biotechnology), from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST, PRIN 1997), from the Italian Association for Cancer Research (AIRC), from the European Commission (Biomed-2), from the Ministero della Sanità (Progetto AIDS, Istituto Superiore di Sanità), from the Armenise-Harvard Foundation and from Polo Nazionale Bioelettronica – Parco Scientifico e Tecnologico dell'Elba. The secretarial aid of Ms. Flavia Bergamin is gratefully acknowledged.

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