Physical Dissection of the Structural Elements Responsible for Regulatory Properties and Intersubunit Interactions of Protein Kinase CK2 β-Subunit†

Oriano Marin,‡,§ Flavio Meggio,‡ Stefania Sarno,‡ and Lorenzo A. Pinna*,‡

Dipartimento di Chimica Biologica, CNR, Centro di Studio delle Biomembrane, Universitá degli Studi di Padova, Padova, Italy, and CRIBI Biotechnology Center, Padova, Italy

Received November 21, 1996; Revised Manuscript Received March 27, 1997⊗

ABSTRACT: The noncatalytic β-subunit of protein kinase CK2 has been shown to display various and in some respects antagonistic effects on the catalytic α-subunit [Boldyreff et al. (1993) *Biochemistry 32*, 12672−12677; Meggio et al. (1994) *Biochemistry 33*, 4336−4342]. We have now examined the ability of peptides encompassing the N- and C-terminal regions of the β-subunit (β[1−77] and β[155−215]) to mimic the functions of the whole-length β-subunit. Peptide β[155−215] possesses only the positive features of the β-subunit in that it prevents thermal inactivation and stimulates basal activity of the α-subunit, while it does not inhibit but rather stimulates calmodulin phosphorylation. In sharp contrast, peptide β[1−77] neither protects the α-subunit nor stimulates its basal activity, while acting as a powerful and specific inhibitor of calmodulin phosphorylation. Peptide β[155−215], but not peptide β[1−77], stably interacts with α-subunit and also displays remarkable self-associating properties. A shorter derivative of β[155−215], β[170−215], displaying weaker stimulatory properties fails to stably interact with the α-subunit and to give rise to dimeric/multimeric forms. These data show that the elements responsible for the negative regulation are concentrated in the N-terminal moiety of the β-subunit, whereas the C-terminal region retains the beneficial properties of the β-subunit and is capable of self-association and binding of the α-subunit. Residues between 155 and 170 are necessary for the latter functions.

It is now generally accepted that nearly all functions in eukaryotes are controlled by a coordinated network of protein phosphorylation events that play a pivotal role in the regulatory machinery of the cell (Marks, 1996). Consequently, the enzymes that catalyze reversible protein phosphorylation, protein kinases (and their counterparts as well, protein phosphatases) are the receivers of an ample variety of signals generated both outside and inside the cell.

Protein kinases belong, with few occasional exceptions, to a single large superfamily of enzymes sharing similar catalytic domains encoded by a substantial amount of the eukaryotic genome (about 2% in *Saccharomyces cerevisiae*). Additional distinctive features, generally present in separate regulatory domains, or sometimes regulatory subunits, are responsible for turning on and off catalytic activity in response to a variety of stimuli. In many instances (e.g., second messenger-dependent protein kinases, receptor tyrosine kinases, cell cycle and mitogen-activated protein kinases) the mechanism of such regulations is fairly well understood.

A notable exception is provided by protein kinase CK2, an essential, ubiquitous, and pleiotropic kinase affecting Ser/Thr residues specified by acidic determinants in a wide variety of protein substrates implicated in many cellular functions, with special reference to gene expression and cell proliferation [reviewed by Pinna (1990), Issinger (1993), and Allende and Allende (1995)]. Despite the fact that CK2 is one of the kinases first discovered (Burnett & Kennedy, 1954) and its quaternary structure, composed of two catalytic (α and/or α′) and two noncatalytic β-subunits, is symptomatic of a regulatable enzyme, its mode of *in vivo* regulation remains enigmatic and merely speculative. The regulatory β-subunits, which, unlike the α/α′ subunits, tend to form homodimers (Kusk et al., 1995; Gietz et al., 1995; Boldyreff et al., 1996), seem to be required for optimal catalytic activity with most CK2 substrates, and no physiological effectors and/or conditions are known that are capable of dissociating the β-subunit from the catalytic ones. With a few substrates, however, exemplified by calmodulin, the activity of CK2 holoenzyme is negligible under basal conditions and the addition of polybasic peptides, such as polylysine, is required in order to elicit phosphorylation (Meggio et al., 1987). It has been shown that, in this case, the β-subunit plays a negative instead of a positive role, by preventing the phosphorylation of calmodulin by the isolated catalytic subunit (Meggio et al., 1992; Bidwai et al., 1993). These observations, in conjunction with mutational studies generating hyperactivating β-subunits (Boldyreff et al., 1993b; Hinrichs et al., 1995) unable to inhibit calmodulin phosphorylation (Meggio et al., 1994), led to the discovery of a dual function of the β-subunit, which behaves as either a stimulatory or an inhibitory element depending on experimental conditions. These latter in fact can favor the display of either the negative or the positive effects of the β-subunit.

These studies suggested that the elements responsible for the positive effects are concentrated in the carboxyl-terminal region of β-subunit, whereas its inhibitory power is mainly due to acidic residues clustered in its N-terminal moiety (Boldyreff et al., 1993b; Meggio et al., 1994; Hinrichs et

† This work was supported by grants from Ministero dell'Universitá e della Ricerca Scientifica e Tecnologica, from Consiglio Nazionale delle Ricerche to L.A.P. (Grant 94.00315.CT14 and Applicazioni Cliniche della Ricerca Oncologica), from the Italian Association for Cancer Research, and from the Ministero della Sanitá (Progetto AIDS, Istituto Superiore di Sanitá).

* Corresponding author: Dipartimento di Chimica Biologica, via Trieste 75, 35121 Padova, Italy. Fax +39 49 8073310. E-mail pinna@civ.bio.unipd.it.

‡ Università di Padova.

§ CRIBI Biotechnology Center.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

S0006-2960(96)02885-1 CCC: $14.00

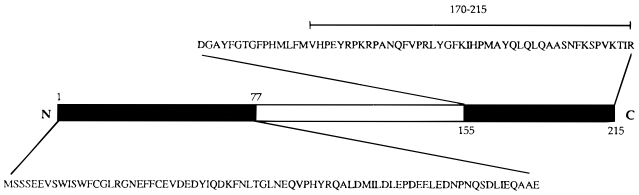

FIGURE 1: Schematic representation of CK2 β-subunit and of its derived β[1−77] and β[155−215] synthetic fragments. A solid line overlaps the C-terminal moiety of β[155−215] contained in the β[170−215] peptide used in a previous study (Marin et al., 1995).

al., 1995; Marin et al., 1995). In order to validate this hypothesis we have now generated two large synthetic peptides encompassing the N- and C-terminal domains of the β-subunit (see Figure 1) and show here that indeed each of them display separate and antagonistic functions (*either* stimulation *or* inhibition), irrespective of the experimental conditions and the use of calmodulin as phosphorylatable substrate.

EXPERIMENTAL PROCEDURES

Materials. Recombinant calmodulin was kindly provided by Professor E. Carafoli (Zürich). MAP kinase p42 was purchased from Santa Cruz Biotechnology. Specific polyclonal antibody anti-human CK2 α-subunit was raised in rabbit against synthetic peptide CVVKILKPVKKKKIKREIKILE, linked to keyhole limpet hemocyanin, corresponding to the 66−86 sequence of the protein. Native protein kinase CK2 (nCK2)[1] was isolated and purified from rat liver cytosol essentially as reported by Meggio et al. (1981) with an additional FPLC/Mono Q chromatographic step. Recombinant human CK2 α and β were prepared as previously described (Grankowski et al., 1991; Boldyreff et al., 1992). Recombinant CK2 holoenzyme (rCK2) was purified after α- and β-expressing bacteria were mixed in a 1:1 ratio according to Boldyreff et al. (1993a).

Peptide Synthesis. Peptide substrate RRRAADSDDDDD was prepared as previously reported (Marin et al., 1994). The peptide VHPEYRPKRPANQFVPRLYGFKIHPMAYQLQLQAASNFKSPVKTIR and its shorter derivative VHPEYRPKRPANQFVPRLYGFKI, reproducing the 170−215 and 170−192 segments of CK2 β-subunit, respectively, were synthesized as previously described (Marin et al., 1995).

The synthesis of 77-mer (β[1−77]) and 61-mer (β[155−215]) peptides was performed using an automatic peptide synthesizer (Model 431 Applied Biosystems) using Fmoc/HBTU chemistry according to previous protocols [for details see Marin et al. (1994, 1995)] adapted to a long synthesis on a 4-(hydroxymethyl)phenoxymethyl−copolystyrene−1% divinylbenzene resin (from Applied Biosystems). The efficiency of the attachment of the first amino acid was reduced in order to lower to 0.3 mmol/g the initial functionalization of the resin that was 0.96 mmol/g. The scale of the synthesis was reduced to 25 μmol. The time of coupling was extended to 90 min using an excess of 10 equiv for Fmoc-amino acids. The monitoring at 301 nm of the removal of Fmoc protection was done after every coupling, while the capping with acetic anhydride was routinely carried out after the introduction of arginine or hydrophobic residues (Val, Leu, Ile, Trp) and during synthesis of troublesome fragments expected according to Milton et al. (1990). The crude peptide after the introduction of the last amino acid was obtained by treatment of the peptidyl-resin with the reagent K (King et al., 1990) (0.1 g of resin in 5 mL of mixture K) and purified by semipreparative reverse-phase HPLC on a C18 column (Delta Pack, Waters, 15 μm, C18, 300 Å, 78 × 300 mm) using a gradient of aqueous TFA (0.1%) and acetonitrile with TFA (0.08%). The homogeneity of the peptides was checked by analytical HPLC (Pep-S, Pharmacia, 5 μm, C2−18, 4.6 × 250 mm) that showed a single symmetrical peak for each peptide, confirming the efficiency of the purification step. Moreover, the mass spectrometry of the purified peptides performed on a MALDI-TOF spectrometer (Model Maldi 1/Kratos-Shimadzu) revealed the correct sequences (8988 and 7098 Da, compared with their calculated masses of 8987.81 and 7097.35 Da for β[1−77] and β[155−215] peptides, respectively) besides minor impurities having a molecular mass very near to the expected peak.

Phosphorylation Assay. Phosphorylation experiments were performed by incubating the phosphorylatable substrate in a medium (25 μL final volume) containing 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 100 mM NaCl, and 20 μM [γ-^{32}P]ATP (specific activity 500−1000 cpm/pmol). The reaction started by adding 2.2 pmol of CK2 α-subunit either alone or previously mixed with equimolar amounts of β-subunit. The reaction was stopped by cooling in ice followed by SDS−PAGE according to Laemmli (1970), staining with Coomassie Brilliant Blue, and either autoradiography or directly scanning on an instant imager apparatus (Canberra-Packard). ^{32}P incorporated into small peptide substrates was evaluated by the phosphocellulose paper procedure (Glass et al., 1978).

Far Western Analysis. Samples of CK2 β-subunit (0.1 nmol) and of β-derived synthetic peptides (2 nmol) were subjected to SDS−PAGE according to Laemmli (1970) and transblotted to nitrocellulose membrane (Bio-Rad) in a Hoefer apparatus at 250 mA for 2.30 h. Membranes were rapidly stained with 0.2% Ponceau in 3% trichloroacetic acid, blocked for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, and then treated as described by Goetz et al. (1996). In particular, the membranes were first equilibrated for 1 h with binding buffer (20 mM Tris-HCl, pH 8.0, containing 300 mM KCl and 0.1% Tween 20) and then gently stirred for 2 h at 4 °C in the same buffer in the presence of CK2 α-subunit (20 μg/mL). Controls were treated with the same buffer but in the absence of α-subunit. Unbound α-subunit was removed by washing the membranes three times with binding buffer. The membranes after incubation with rabbit polyclonal anti-α antibody were treated with biotinylated donkey anti-rabbit IgG, and bound α was detected by incubation with streptavidin−alkaline phosphatase conjugate.

Sucrose Density Gradient Analysis. Linear gradients (5 mL) of 5−20% (w/v) sucrose were prepared in 25 mM Tris-HCl, pH 8.5, containing 300 mM NaCl. Protein samples (100 μL) were loaded on top of the gradient and centrifuged at 38 000 rpm in a Beckman SW39 rotor for 16 h. Twenty fractions were collected and monitored by either silver-staining procedure and densitometric scanning of the gels. Carbonic anhydrase (29 kDa) and bovine serum albumin (68

[1] Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; rCK2, recombinant CK2 holoenzyme; nCK2, native CK2 holoenzyme purified from rat liver.

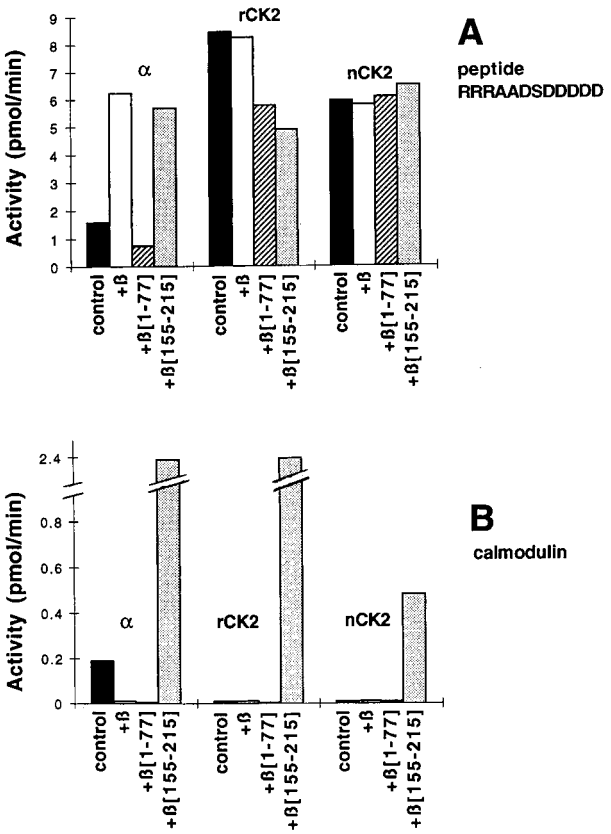

FIGURE 2: Variable effect of peptides β[1−77] and β[155−215] on CK2 catalytic activity tested either with a canonical peptide substrate or with calmodulin. Phosphorylation conditions and evaluation of the phosphate incorporated were described under Experimental Procedures. The activities of recombinant α-subunit (2.2 pmol), recombinant CK2 (0.7 pmol), and native CK2 (2.1 pmol) were assayed by using either 200 μM synthetic peptide RRRAADSDDDDD (panel A) or 10 μM calmodulin (panel B) in the absence or in the presence of the indicated synthetic fragments (40 μM). β-subunit was added at 1:1 molar ratio with respect to the α-subunit. The data are the average of at least 3 separate experiments with a standard error ≤ 10%. Similar results were obtained if NaCl was omitted in the incubation medium and by varying the concentration of the peptide substrate (50 and 500 μM) and of calmodulin (5 and 26 μM).

kDa) were run separately at the same conditions as molecular mass markers.

RESULTS

The β-subunit has been shown to exert a dual effect on the catalytic activity of protein kinase CK2, resulting in net inhibition or stimulation of phosphorylation rate depending on experimental conditions and substrate used (Meggio et al., 1992; Tiganis et al., 1993). With most, if not all, peptide substrates a more or less pronounced stimulation is observed, interpreted as predominance of positive over negative regulation. By contrast to these "canonical" substrates, calmodulin phosphorylation invariably undergoes drastic inhibition by the β-subunit (Meggio et al., 1992). These diametrically opposed effects of the β-subunit are illustrated in Figure 2, showing that while the phosphorylation of the specific peptide substrate RRRAADSDDDDD is increased by adding equimolar amounts of the β-subunit to the catalytic subunit (Figure 2A), the same addition suppresses the phosphorylation of calmodulin (Figure 2B).

If however, instead of using the whole β-subunit, its synthetic amino- and carboxyl-terminal regions, spanning residues 1−77 and 155−215, respectively (see Figure 1), are added to the catalytic subunit, the opposite behavior with the two substrates disappears. As also shown in Figure 2, in fact, the N-terminal peptide is inhibitory with both the peptide substrate and calmodulin, whereas the C-terminal peptide stimulates the phosphorylation of both the peptide substrate and calmodulin. Not only inhibition by β[1−77] but also stimulation by β[155−215] actually is more pronounced with calmodulin, supporting the view that calmodulin is not refractory to positive regulation by elements residing in the C-terminal moiety of the β-subunit, albeit this effect is normally overridden by the prevailing negative regulation imposed by the N-terminal domain of β.

If, instead of the isolated α-subunit, the holoenzyme, composed of two catalytic and two β-subunits, is used for similar experiments with peptide substrate, neither the addition of extra β-subunit nor that of the synthetic peptides β[1−77] and β[155−215] causes the marked effects observed with α alone (Figure 2A). With calmodulin, whose phosphorylation by CK2 holoenzyme is negligible due to the prevailing inhibitory effect of the β-subunit, the β[1−77] peptide has no effect while the C-terminal β[155−215] peptide triggers a remarkable phosphorylation of calmodulin by CK2 holoenzyme. Significant differences are observed using either the recombinant or the native holoenzymes, the former being more susceptible to the opposite effects exerted by β[155−215] on peptide substrate (panel A) and calmodulin (panel B) phosphorylation. This would indicate that the quaternary structure of native CK2 is tighter and/or more stable than that of reconstituted CK2, possibly due to posttranslational modifications and/or chaperonine-assisted folding occurring in the heptocyte where native CK2 is produced.

The inhibitory peptide β[1−77] includes the autophosphorylation site of the β-subunit (Ser-2/Ser-3) (Litchfield et al., 1991; Boldyreff et al., 1993a, 1994; Bodenbach et al., 1994; Lin et al., 1994) in its N-terminal end. It was possible, therefore, that inhibition of catalytic activity by this peptide was merely accounted for by substrate competition. Several observations, however, argue against this interpretation and support the view that peptide β[1-77], similar to peptide β-[155−215], operates by mimicking some specific functions of the β-subunit: (i) As just pointed out, the efficacy of β-[1−77] is only remarkable with the catalytic subunit alone, becoming negligible with the holoenzyme, where the active site is accessible to substrates though the catalytic subunits are already combined with the β-subunits. (ii) The extent of inhibition is crucially dependent on the phosphorylatable substrates used for monitoring CK2 activity. As previously shown (Meggio et al., 1992; Pinna et al., 1992) CK2 substrates are variably susceptible to polylysine stimulation, which is mediated by the β-subunit, and whose extent appears to parallel the predominance of negative over positive effects of the β-subunit (Meggio et al., 1994; Marin et al., 1995). As shown in Table 1 the phosphorylation of calmodulin, a polylysine-dependent substrate *par excellence*, is by far more susceptible to β[1−77] inhibition than that of casein, whose phosphorylation by CK2 holoenzyme is only moderately stimulated by polylysine, and the phosphorylation of inhibitor 2, which is insensitive to polylysine (Pinna et al., 1992), is not significantly affected by β[1−77] up to 10 μM. (iii) Inhibition of calmodulin phosphorylation by β[1−77] is not

Table 1: IC_{50} Values for Inhibition by β[1−77] of CK2 α Activity Tested with Different Protein Substrates[a]

subtrate	IC_{50} (μM)
I-2 (2.7 μM)	>10.00
casein (5 μM)	6.00
calmodulin (5 μM)	0.45
calmodulin (10 μM)	0.42
calmodulin (26 μM)	0.65

[a] Phosphorylation conditions and evaluation of the phosphate incorporated into different substrates were described under Experimental Procedures. All substrates were tested at concentrations lower than the corresponding K_m values (4, 14, and 26 μM for inhibitor 2, casein, and calmodulin, respectively).

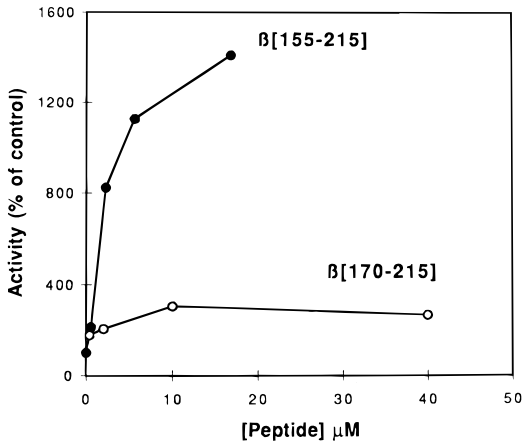

FIGURE 3: Phosphorylation of calmodulin by CK2 α-subunit is stimulated to different extents by C-terminal fragments β[155−215] and β[170−215]. Calmodulin (10 μM) was phosphorylated by CK2 α-subunit (2.2 pmol) either in the absence or in the presence of increasing concentrations of peptides β[170−215] and β[155−215] and the phosphate incorporated was evaluated as described under Experimental Procedures. NaCl was omitted in the incubation medium.

counteracted by increasing calmodulin concentration, as would be expected if it were a merely competitive inhibition (Table 1).

We have previously shown that significant stimulation of CK2 α activity can be promoted by C-terminal β peptides shorter than the ones used in the present study, namely, β-[170−215] and β[170−192] (Marin et al., 1995). As shown in Figure 3 however, the stimulatory efficiency of β[155−215] is by far superior, indicating that the segment between residues 155 and 170 must contain elements that are essential for high-affinity binding to and stimulation of the α-subunit.

The actual implication of the 155−171 segment in high-affinity binding to the α-subunit is highlighted by the far western blot experiments shown in Figure 4 showing that strong signals of α-subunit were detected in the lanes of the whole β-subunit and of the β[155−215] peptide. The shorter C-terminal peptide, β[170−215], gives no detectable signal and no evidence at all of bound α-subunit was detectable in the lanes of the N-terminal peptide β[1−77] and of the shortest C terminal peptide, β[170−192], as well. Interestingly, moreover, in the lane of β[155−215] two immunoreactive bands are detectable; the minor one corresponds to the main protein band of the peptide (apparent $M_1 = 7090$), while a more intense immunoreactivity coincides with a faint stained band whose apparent M_r is twice that of β[155−215] (see Figure 4).

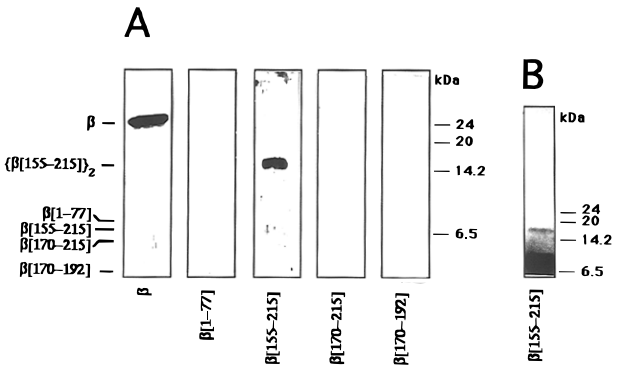

FIGURE 4: Far Western blots revealing interactions of CK2 α-subunit with CK2 β-subunit and its fragments. (Panel A) Immunodetection of bound α-subunit after SDS−18% PAGE and transblotting performed as detailed under Experimental Procedures. (Panel B) Ponceau-stained bands of a parallel experiment where β[155−215] was run on SDS−15% PAGE and transblotted. The presence in the β[155−215] lane of the upper band with M_r = 14 800 was confirmed in parallel experiments by Coomassie staining and by using β[155−215] phosphoradiolabeled by incubation with ^{32}P[ATP] and MAP kinase (see Discussion).

Actually, the tendency of β[155−215] to homodimerize seems to be more pronounced than that of the β-subunit itself, since no evidence for β dimers can be seen on SDS−PAGE where β[155−215] dimers are detectable. Under less dissociating conditions, as in the case of sucrose gradient ultracentrifugation, in the absence of detergents, the β-subunit alone exhibits the apparent M_r expected for its dimer (Figure 5A), whereas the β[155−215] peptide gives rise to component-(s) whose sedimentation coefficient is much higher than that of the whole β-subunit, suggesting that large polymers are formed (Figure 5B). If the β-subunit is mixed with the β-[155−215] peptide, it migrates toward the bottom of the tube together with β[155−215], consistent with the formation of heteropolymers composed by β and β[155−215] (see Figure 5C). Heteropolymers are also formed if reconstituted CK2 holoenzyme is mixed with the β[155−215] peptide. As shown in Figure 5D, these heteropolymers contain both α- and β-subunits and the β[155−215] peptide. They also account for the whole catalytic activity recovered after ultracentrifugation (not shown). These data corroborate the view that the C-terminal domain of the β-subunit contains highly (self)adhesive elements, whose "sticky" effect is attenuated by other domains of the β-subunit.

DISCUSSION

Downregulation of protein kinases is often imposed by inhibitory subunits (e.g., PKA) or domains (e.g., PKG, PKC, CaM kinases) operating as pseudosubstrates and whose negative effect is removed by physiological activators, notably by second messengers [reviewed by Soderling (1990)]. In the case of cyclin-dependent protein kinases (cdks) the regulatory subunit, cyclin, conversely plays a positive role, representing a necessary, albeit not a sufficient, condition for activity (Fisher & Morgan, 1994; Jeffrey et al., 1995).

In this respect CK2 is unique since its regulatory subunit exerts a dual function, playing at the same time positive and negative roles. The first reconstitution experiments with either native renatured (Cochet & Chambaz, 1983) or recombinant subunits (Grankowski et al., 1991; Filhol et al., 1991; Jakobi & Traugh, 1992; Birnbaum et al., 1992)

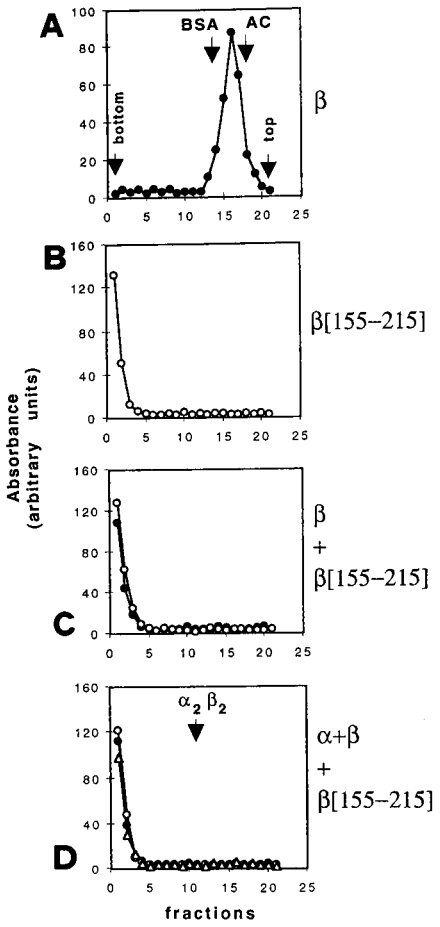

FIGURE 5: Sucrose gradient ultracentrifugation of CK2 β-subunit and of its 155−215 C-terminal fragment. Ultracentrifugation was performed as described under Experimental Procedures. The concentration of α- and β-subunits was 4.5 μM; the concentration of β[155−215] was 45 μM. In the experiment of panel D, CK2 holoenzyme was reconstituted prior to ultracentrifugation by mixing α- and β-subunits before addition of β[155−215]. Aliquots of the fractions collected after ultracentrifugation were subjected to SDS−PAGE and the gels were silver-stained. The 25-, 7-, and 45-kDa bands corresponding to the β-subunit (●), its C-terminal fragment (○), and α-subunit (△), respectively, were quantified after densitometric scanning. The position migrated by CK2 holoenzyme alone is indicated by the arrow. The catalytic activity of CK2 holoenzyme mixed with β[155−215] was fully recovered in the bottom fractions together with α-subunit (not shown). The positions migrated by carbonic anhydrase (AC) and bovine serum albumin (BSA) are indicated in panel A.

outlined only the positive effects of the β-subunit, supporting the view that, as in the case of cyclins with cdks, which belong to the same kinase subfamily as CK2 (Hanks & Quinn, 1991), this noncatalytic subunit was a stimulatory element, required for optimal activity of CK2. Subsequently, however, additional evidence accumulated, based on mutations of the β-subunit (Boldyreff et al., 1993b), use of different substrates (Meggio et al., 1994), and variable experimental conditions (Tiganis et al., 1993), suggesting that, besides its positive functions, the β-subunit also exerts a downregulation of catalytic activity, which, under certain conditions, can become predominant. Generally speaking, the best conditions for highlighting the positive role of the β-subunit are to test its activity on specific peptide substrates and in the presence of 100−200 mM NaCl, which stimulates the tetrameric holoenzyme while inhibiting the isolated α-subunit; conversely, the negative function of the β-subunit is magnified if the substrate is calmodulin, whose phosphorylation by CK2 α is invariably inhibited by equimolar amounts of the β-subunit.

Table 2: Synopsis of the Functional Properties of β-Subunit and Its Synthetic Amino- and Carboxyl-Terminal Domains

	whole β	β[1−77]	β[155−215]	β[170−215]
stimulation of α basal activity (peptide substrate)	+++		++	+
inhibition of α basal activity (peptide substrate)		+		
inhibition of CaM phosphorylation	+++	++		
stimulation of CaM phosphorylation			++	+
α protection against denaturation	+++		++	++
physical interaction with α	+++		++	+/−
self-association	++		+++	−

The complete physical dissection of these two putative functional domains has now been achieved by constructing two synthetic peptides encompassing the 1−77 and 155−215 sequences of β-subunit and by comparing their properties with those of the full-length recombinant β-subunit.

The main outcomes of this study are summarized in Table 2. They are based on the following observations: (i) The N-terminal β[1−77] peptide retains the inhibitory properties of the β-subunit (especially evident using calmodulin as phosphorylatable substrate) without exhibiting any of its positive effects nor its propensity to stably interact with the α-subunit. (ii) Inhibition by β[1−77] is counteracted by the whole β-subunit. (iii) In sharp contrast to β[1−77], the peptide β[155−215], reproducing the whole C-terminal sequence, displays the positive but not the negative effects of the β-subunit: it not only protects against thermal inactivation (unpublished data) and stimulates basal activity toward canonical peptide substrates but also increases the activity of α toward calmodulin, a substrate whose phosphorylation is prevented by the whole β-subunit. (iv) The C terminal β[155−215] peptide also strongly interacts with the α-subunit as judged from far western blot analysis. (v) The β[155−215] peptide tends to self-assemble and to interact with both full-length β and CK2 holoenzyme. In particular, a dimeric form of β[155−215] partially survives denaturing conditions of SDS−PAGE (Figure 4). Under nondenaturing conditions, β[155−215] forms larger polymers that tend to aggregate also β-subunit and CK2 holoenzyme, as disclosed by sucrose gradient ultracentrifugation (Figure 5). (vi) The SDS−PAGE-resistant dimeric form of β[155−215] is more prone to interaction with α than monomeric β[155−215], consistent with the concept that homodimerization of β facilitates its interaction with α. (vii) An N-terminally truncated form of the β[155−215] peptide, β-[170−215], is much less stimulatory of catalytic activity and devoid of detectable self-associating and α-interacting properties, suggesting that the 155−170 sequence contains elements which are important for both tight physical association and stimulation of catalytic activity.

Since peptide β[155−215] includes the phosphoacceptor site affected by cdc2 and MAP kinase (Ser 209), it was interesting to check if this phosphorylation alters the functional properties outlined above. The results of experiments run with peptide β[155−215] phosphoradiolabeled by incubation with MAP kinase and [γ-^{32}P]ATP support the

notion that phosphorylation does not significantly influence either stimulation of catalytic activity or capability to dimerize and to associate with the α-subunit (not shown). The radiolabeled peptide, moreover, once subjected to SDS−PAGE and autoradiographed, showed a major band corresponding to the main stained band of M_r 7090 and a minor one corresponding to M_r 14 800, similar to the nonphosphorylated peptide (Figure 4B).

Despite the tight parallelism between conditions ensuring β−α interactions and stimulation of catalytic activity outlined above (points iii, iv, and vii), the question whether physical interaction and activation occur through identical mechanisms remains unanswered. Expectedly, α−β interaction is a prerequisite for efficient stimulation of activity; it may represent, however, a necessary yet not sufficient condition for it. Likewise, it is not entirely clear which is the role, if any, of β homodimerization in β−α interactions; our far western blot experiments support the view that β homodimerization, if not an absolute prerequisite, certainly is a condition that favors β−α interaction.

Our finding that the 155−172 sequence is critical not only for stimulation of catalytic activity and β−α interaction but also for tight β−β contacts is in good agreement with the outcome of 2H system experiments highlighting the importance of residues between 156 and 165 for β homodimerization (Boldyreff et al., 1996). These studies also led to the conclusion that the tightness of this interaction is attenuated by residues between 170 and 180 (Boldyreff et al., 1996). From our data, showing that β[155−215] tends to dimerize/oligomerize more markedly than full-length β-subunit, we have to assume that additional residues capable of antagonizing β self-assembly are also present upstream from residue 155. A good candidate for such a role appears to be the N-terminal region comprising peptide β[1−77], where the predominant negatively charged residues are expected to generate repulsive interactions between β-subunits. This would also be consistent with the lack of any detectable self-association of the peptide β[1−77] as opposed to β[155−215].

The electrostatic nature of the interactions responsible for negative regulation would be consistent with the failure to detect stable interactions between β[1−77] and α in media containing salt and/or SDS. It is also in agreement with the finding that the inhibitory power of β[1−77] is lower than that of full-length β-subunit, which is capable of preventing calmodulin phosphorylation by α-subunit at equimolar concentration, whereas a more than 20-fold higher concentration of β[1−77] is required to reach the same effect. It is conceivable, therefore, that the juxtaposition of the N-terminal inhibitory domain of β with its counterpart in the α-subunit is facilitated by the physical nonelectrostatic interactions mediated by the C-terminal β domain. Consistent with this view, the β[155−215] C-terminal peptide deeply affects the interactions normally occurring between α and the N-terminal region of β, as disclosed by its capability to overcome inhibition of calmodulin phosphorylation and intramolecular phosphorylation of β at its N-terminal end (unpublished data).

Such a situation, where a physical association domain is distinct from the pseudosubstrate domain, would be reminiscent of the regulatory subunits (RI and RII) of PKA (Taylor, 1989; Cox & Taylor, 1994). In the case of CK2, however, no physiological effector, comparable to cAMP for PKA, is known which is capable of dissociating the regulatory subunits from the catalytic ones. Interestingly, it has been recently shown that activation of PKA by cAMP does not necessarily imply complete release of the regulatory subunit but can also occur through the weakening of interactions between the pseudosubstrate domain and the active site of the catalytic subunit (Yang et al., 1995). It may be worthwhile to further investigate the possibility that physiological effectors/conditions may exist capable of overcoming the negative regulation imposed by the β-subunit on the catalytic subunit of CK2 similar to the in vitro effect of polybasic peptides and β[155−215]. Their discovery would represent a critical breakthrough into the understanding of the mechanism(s) that control CK2 activity in the intact cell, which presently are still obscure.

REFERENCES

Allende, J. E., & Allende, C. C. (1995) *FASEB J. 9*, 313−323.

Bidwai, A. P., Reed, J. C., & Glover, C. V. C. (1993) *Arch. Biochem. Biophys. 300*, 265−270.

Birnbaum, M. J., Wu, J., O'Reilly, D. R., Rivera-Marrero, C. A., Hanna, D. E., Miller, L. K., & Glover, C. V. C. (1992) *Protein Expression Purif. 3*, 142−150.

Bodenbach, L., Fauss, J., Robitzki, A., Krehan, A., Lorenz, P., Lozeman, F. J., & Pyerin, W. (1994) *Eur. J. Biochem. 220*, 263−273.

Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1992) *Biochem. Biophys. Res. Commun. 188*, 228−234.

Boldyreff, B., James, P., Staudenmann, W., & Issinger, O.-G. (1993a) *Eur. J. Biochem. 218*, 515−521.

Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1993b) *Biochemistry 32*, 12672−12677.

Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1994) *J. Biol. Chem. 269*, 4827−4832.

Boldyreff, B., Mietens, U., & Issinger, O.-G. (1996) *FEBS Lett. 379*, 153−156.

Burnett, G., & Kennedy, E. P. (1954) *J. Biol. Chem. 211*, 969−980.

Cochet, C., & Chambaz, E. M. (1983) *J. Biol. Chem. 258*, 1403−1406.

Cox, S., & Taylor, S. S. (1994) *J. Biol. Chem. 269*, 22614−22622.

Filhol, O., Cochet, C., Wedegaertner, P., Gill, G. N., & Chambaz, E. M. (1991) *Biochemistry 30*, 11133−11140.

Fisher, R. P., & Morgan, D. O. (1994) *Cell 78*, 713−724.

Gietz, D., Graham, K. C., & Litchfield, D. W. (1995) *J. Biol. Chem. 270*, 13017−13021.

Glass, D. B., Masaracchia, R. A., Feramisco, J. R., & Kemp, B. E. (1978) *Anal. Biochem. 87*, 566−575.

Goetz, C., Wagner, P., Issinger, O.-G. & Montenarh, M. (1996) *Oncogene 13*, 391−398.

Grankowski, N., Boldyreff, B., & Issinger, O.-G. (1991) *Eur. J. Biochem. 198*, 25−30.

Hanks, S. K., & Quinn, A. M. (1991) *Methods Enzymol. 200*, 38−62.

Hinrichs, M. V., Gatica, M., Allende, C. C., & Allende, J. E. (1995) *FEBS Lett. 368*, 211−214.

Issinger, O.-G. (1993) *Pharmacol. Ther. 59*, 1−30.

Jakobi, R., & Traugh, J. A. (1992) *J. Biol. Chem. 267*, 23894−23902.

Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massagué, J., & Pavletich, N. P. (1995) *Nature (London) 376*, 313−320.

King, D. S., Fields, C. G., & Fields, G. B. (1990) *Int. J. Pept. Protein Res. 36*, 255−266.

Kusk, M., Bendixen, C., Duno, M. Westergaard, O., & Thomsen, B. (1995) *J. Mol. Biol. 253*, 703−711.

Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.

Lin, W.-J., Sheu, G.-T., & Traugh, J. A. (1994) *Biochemistry 33*, 6998−7004.

Litchfield, D. W., Lozeman, F. J., Cicirelli, M. F., Harrylock, M., Ericsson, L. H., Piening, C. J., & Krebs, E. G. (1991) *J. Biol. Chem. 266*, 20380−20389.

Marin, O., Meggio, F., & Pinna, L. A. (1994) *Biochim. Biophys. Res. Commun. 198*, 898−905.
Marin, O., Meggio, F., Boldyreff, B., Issinger, O.-G., & Pinna, L. A. (1995) *FEBS Lett. 363*, 111−114.
Marks, F. (1996) in *Protein Phosphorylation* (Marks, F., Ed.) pp 1−35, VCH Weinheim, New York, Basel, Cambridge, and Tokyo.
Meggio, F., Donella Deana, A., & Pinna, L. A. (1981) *J. Biol. Chem. 256*, 11958−11961.
Meggio, F., Brunati, A. M., & Pinna, L. A. (1987) *FEBS Lett. 160*, 203−308.
Meggio, F., Boldyreff, B., Marin, O., Marchiori, F., Perich, J. W., Issinger, O.-G., & Pinna, L. A. (1992) *Eur. J. Biochem. 205*, 939−945.
Meggio, F., Boldyreff, B., Issinger, O.-G., & Pinna, L. A. (1994) *Biochemistry 33*, 4336−4342.
Milton, R. C. de L., Milton, S. C. F., & Adams P. A. (1990) *J. Am. Chem. Soc. 112*, 6039−6046.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267−284.
Pinna, L. A., Meggio, F., & Perich, J. W. (1992) in *Recent Advances in Cellular and Molecular Biology* (Wegmann, R. J., & Wegmann, M. A., Eds.) Vol. 4, pp 23−27, Peeters Press, Leuven, Belgium.
Soderling, T. R. (1990) *J. Biol. Chem. 265*, 1823−1826.
Taylor, S. S. (1989) *J. Biol. Chem. 264*, 8443−8446.
Tiganis, T., House, C. M., & Kemp, B. E. (1993) *Biochim. Biophys. Acta 1203*, 282−289.
Yang, S., Fletcher, W. H., & Johnson, D. A. (1995) *Biochemistry 34*, 6267−6271.

BI962885Q