Casein Kinase 2 Down-Regulation and Activation by Polybasic Peptides Are Mediated by Acidic Residues in the 55–64 Region of the β-Subunit. A Study with Calmodulin As Phosphorylatable Substrate†

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ABSTRACT: The noncatalytic β-subunit is responsible for the latency of casein kinase 2 (CK2) activity toward calmodulin. Twenty-one mutants of the β-subunit bearing either deletions or Ala substitutions for charged residues in the 5–6, 55–70, and 171–178 sequences have been assayed for their ability to substitute for wild-type β-subunit as a suppressor of activity toward calmodulin. The only mutations that reduced the ability of the β-subunit to suppress calmodulin phosphorylation activity, though being compatible with normal reconstitution of CK2 holoenzyme, were those affecting Asp^{55}, Glu^{57} and the whole triplet Glu^{59}-Asp-Glu^{61}. The activity of CK2 holoenzyme, either native or reconstituted, toward calmodulin can be elicited by a variety of polybasic effectors, including polylysine, polyarginine, salmine, and histones H4, H3, and, to a lesser extent, H2a and H2b. Histone H1 and polyamines are conversely ineffective. The latent "calmodulin kinase" activity of CK2 can also be specifically unmasked by a peptide (α[66–86]) reproducing a basic insert of the catalytic subunit. This effect is reversed by equimolar addition of a peptide (β[55–71]) including the 55–64 acidic stretch of the β-subunit. Comparable polylysine stimulation was observed with the holoenzymes reconstituted with either β^{wt} or the β mutants capable of assembling with the α-subunit, with the notable exception of those bearing Ala substitutions for acidic residues at positions 55, 57, and 59–61. These were nearly insensitive to 42 nM polylysine, which conversely promotes a more than 10-fold increase of calmodulin phosphorylation with wild-type β. Together, these results show that the negative control of the β-subunit over CK2 activity toward calmodulin is crucially dependent on the integrity of an acidic cluster in the 55–64 sequence which is also primarily responsible for the specific responsiveness to polycationic effectors.

Casein kinase 2 (CK2)[1] is a conventional name applied to a ubiquitous acidophilic Ser/Thr-specific protein kinase whose early detection in a variety of organisms and tissues using casein and phosvitin as artificial substrates was facilitated by its constitutive basal activity [reviewed by Pinna (1990) and Tuazon and Traugh (1991)]. This feature of being apparently independent, at least *in vitro*, of any strict control mechanism is hardly compatible with the central role CK2 is believed to play in cellular regulation (Issinger, 1993). The list of physiological targets of CK2 is large and includes a variety of proteins involved in many different biological functions, e.g., gene expression, cell proliferation, and signal transduction. This would obviously imply that CK2 activity is subjected to some kind of modulation. Despite many efforts, however, the mechanism(s) by which CK2 activity is regulated *in vivo* is still a matter of conjecture. Special attention has been devoted to the quaternary structure of CK2, which is composed of two noncatalytic β-subunits tightly associated with two catalytic α- and/or α′-subunits to give heterotetramers, which apparently do not dissociate except under denaturing conditions. Although the β-subunit is required in order to stabilize the enzyme (Meggio et al., 1992a) and to optimize its activity toward most of its substrates (Cochet & Chambaz, 1983; Grankowski et al., 1991; Filhol et al., 1991; Birnbaum et al., 1992), we have recently shown by site-directed mutagenesis that an acidic region of the β-subunit imposes a negative control on CK2 activity (Boldyreff et al., 1992). While such an intrinsic down-regulation is still compatible with the efficient phosphorylation of many substrates, it could be at least partially responsible for the observed inability of the CK2 holoenzyme to phosphorylate calmodulin under basal conditions (Meggio et al., 1992b). Calmodulin, whose seryl and threonyl residues that are affected *in vitro* by CK2 (Meggio et al., 1987; Sacks et al., 1992) are also phosphorylated *in vivo* (Sacks et al., 1992), is a peculiar substrate of CK2 in two respects: (i) It can be phosphorylated by the isolated recombinant catalytic subunit of CK2 but not by the native enzyme nor by the recombinant reconstituted $\alpha_2\beta_2$ tetramer, under basal conditions (Meggio et al., 1992b; Bidwai et al., 1993). (ii) It belongs to a small group of CK2 substrates, including, among others, ornithine decarboxylase (Meggio et al., 1984), whose phosphorylation by the CK2 holoenzyme is entirely dependent on the addition of polybasic peptides like

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[1] Abbreviations: CaM, calmodulin; CK2, casein kinase 2; β^{wt}, β-subunit, wild type; $rCK2^{wt}$, CK2 holoenzyme reconstituted by mixing *in vitro* the α- and the β-subunit expressed in bacteria; α-peptide, synthetic 66–86 fragment of α-subunit; β-peptide, synthetic 55–71 fragment of β-subunit.

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polylysine. These polycations, which require the β-subunit to achieve their maximal effectiveness (Lin et al., 1991; Meggio et al., 1992b), also increase the phosphorylation of many other CK2 protein substrates to a variable extent. On the other hand a new insight into the β-subunit functional domains was recently provided by mutagenesis experiments (Boldyreff et al., 1992, 1993). These studies showed that a C-terminal segment is essential for holoenzyme reconstitution. A number of acidic residues clustered in the 55–64 sequence which are conserved in all known β-subunits from different species are responsible for an intrinsic down-regulation of CK2 basal activity. In fact, their substitutions with alanyl residues promoted an increased activity toward canonical substrates (Boldyreff et al., 1993). Interestingly enough, these acidic residues are deleted in the only known protein homologous to the β-subunit, i.e., the product of the stellate gene, responsible for the appearence of proteinaceous star-shaped crystals in the primary spermatocytes of *Drosophila melanogaster* males lacking a Y chromosome (Livak et al., 1990). The stellate protein is unable to reconstitute functional CK2 holoenzyme by combining with the α-subunit (M. P. Bozzetti, F. Meggio, S. Pimpinelli, and L. A. Pinna, unpublished results), and it is divergent from the β-subunit in two regions, namely, the C-terminal tail, which is essential for high-affinity binding of the β-subunit to the α-subunit, and the 55–64 acidic stretch, which is responsible for down-regulation (Boldyreff et al., 1993). These observations prompted a study aimed at assessing whether charged residues present in these regions are also responsible for the lack of CK2 basal activity toward calmodulin and for the triggering of such an activity by polybasic effectors.

MATERIALS AND METHODS

Materials. Calmodulin was kindly provided by Professor E. Carafoli (Zürich). Polylysine and polyarginine (average molecular weight 47 500 and 40 000, respectively) were from Sigma. Histones were from Boehringer Mannheim. Salmine and clupeine were a gift of Professor F. Marchiori.

The peptides CVVKILKPVKKKKIKREIKILE, DLEPDEELEDNPNQSDL, and RKMKDTDSEEEIR, reproducing the 66–86 fragment of the α-subunit (α-peptide), the 55–71 fragment of the β-subunit (β-peptide), and the 74–86 fragment of calmodulin, respectively, and the peptide RRRDDDSDDD were synthesized by a continuous-flow solid-phase method using an automatic synthesizer (Model 431A, Applied Biosystems). Recombinant CK2 α-subunit was prepared as described by Grankowski et al. (1991). Its specific activity was 52 units/mg, 1 unit being defined as the catalytic activity transferring 1 nmol of P per min into casein (80 μM) under standard conditions. Native CK2 was purified from rat liver cytosol as previously described (Meggio et al., 1981) with a subsequent FPLC Mono Q chromatographic step. Its specific activity was 236 units/mg.

Cloning and Expression of Mutants. Mutants with deletions at the N- or C-terminus were created by a PCR-based method by Boldyreff et al. (1992, 1993) using the expression plasmid pBB4 (Grankowski et al., 1991) as a template.

Mutated CK2 β-subunits were expressed as described previously (Grankowski et al., 1991). All mutants were purified to >95% homogeneity as judged by Coomassie blue staining as described in Boldyreff et al. (1992, 1993). The list of the mutants used in this study is provided in Table 1.

Phosphorylation Assays. Calmodulin (10 μM) was phosphorylated for 10 min in a medium containing 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 20 μM [γ^{32}P]ATP (specific activity, 1000–2000 cpm/pmol), and 50–150 ng of α-subunit either alone or in combination with equimolar amounts of various β-mutants. The reaction was stopped by cooling in ice, and the samples were subjected to SDS–PAGE according to Laemmli (1970). The gels were dried and autoradiographed, and the radioactive calmodulin bands were excised and counted in a liquid scintillation counter (Packard). The phosphorylation of the synthetic peptide RKMKDTDSEEEIR (0.5 mM) was performed as described above for calmodulin and evaluated by the phosphocellulose paper procedure (Glass et al., 1978).

Phosphoamino Acid Analysis. Radioactive samples of calmodulin, phosphorylated as described above, were eluted from the stained gels and digested with 6 N HCl at 105 °C for 4 h. The subsequent isolation of phosphoserine and phosphothreonine by high-voltage paper electrophoresis was performed as previously described (Perich et al., 1992).

Table 1: Inhibition of "Calmodulin Kinase" Activity of CK2 Catalytic Subunit by Equimolar Amounts of Wild-Type and Mutated β-Subunits

subunit[a]	mutation(s)	inhibition[b] (%)
β wild type		91
$\beta A^{5,6}$	Glu^5 and Glu^6 replaced by Ala	93
βA^{55-57}	Asp^{55}, Leu^{56}, and Glu^{57} replaced by Ala	28
$\beta A^{55,57}$	Asp^{55} and Glu^{57} replaced by Ala	30
βA^{55}	Asp^{55} replaced by Ala	24
βA^{57}	Glu^{57} replaced by Ala	24
βA^{59-61}	Asp^{59}, Glu^{60}, and Glu^{61} replaced by Ala	48
$\beta A^{59-61,63,64}$	Asp^{59}, Glu^{60}, Glu^{61}, Glu^{63}, and Asp^{64} replaced by Ala	23
$\beta A^{63,64}$	Glu^{63} and Asp^{64} replaced by Ala	94
βA^{63}	Glu^{63} replaced by Ala	85
βA^{64}	Asp^{64} replaced by Ala	85
βA^{70}	Asp^{70} replaced by Ala	72
βA^{171}	His^{171} replaced by Ala	87
βA^{173}	Glu^{173} replaced by Ala	89
βA^{175}	Arg^{175} replaced by Ala	90
$\beta A^{177,178}$	Lys^{177} and Arg^{178} replaced by Ala	88
β1–4	deletion of residues 1–3 and Ser^4 replaced by Met	94
β209–215	deletion of residues 209–215	85
β194–215	deletion of residues 194–215	90
β181–215	deletion of residues 181–215	30
β171–215	deletion of residues 171–215	7
β150–215	deletion of residues 150–215	5

[a] Nomenclature of mutated β-subunits is indicative of either the segment that has been deleted (preceded by Δ) or the position(s) (denoted by superscripts) where the mutated residues have been replaced by Ala. [b] Data drawn from Figure 2 and from parallel experiments performed as described under Materials and Methods.

RESULTS

Inhibition of Calmodulin Phosphorylation by the β-Subunit of CK2 and by a β-Subunit Peptide. It has been shown previously (Meggio et al., 1992b) that under basal conditions (i.e., in the absence of polybasic effectors), in contrast to the isolated catalytic subunit, the CK2 holoenzyme is unable to phosphorylate calmodulin. This negative control of calmodulin phosphorylation by the noncatalytic β-subunit is highlighted by the experiment shown in Figure 1A. Like that toward the majority of CK2 targets (Meggio et al., 1992b), the activity toward a calmodulin-derived peptide increases during the gradual reconstitution of the CK2 holoenzyme following the addition of the β- to the α-subunit. In contrast, the activity toward calmodulin is abolished. Both the maximal activity toward the peptide and the disappearance of activity toward calmodulin occur with a 1:1 molar ratio of α to β, which gives rise to canonical $\alpha_2\beta_2$ tetramers (Grankowski et al., 1991).

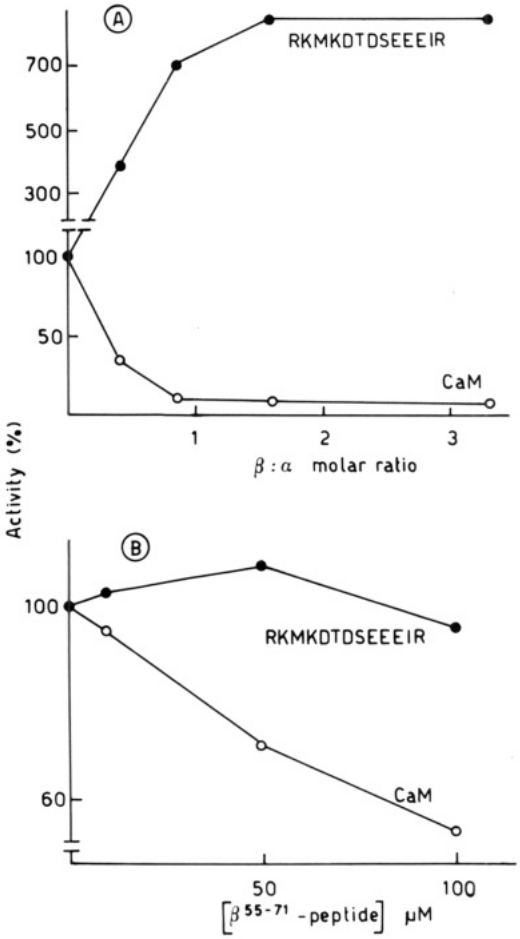

FIGURE 1: Down-regulation of calmodulin phosphorylation upon addition of either the β-subunit (A) or the β-peptide (B) to the catalytic (α) subunit of CK2. Calmodulin phosphorylation is denoted by open circles (O). For comparison, the phosphorylation of a peptide representative of canonical CK2 substrates is also shown (●). Phosphorylation of calmodulin and of the peptide by CK2 α-subunit either alone or in combination with increasing concentrations of the β-subunit was performed and evaluated as described under Materials and Methods.

As shown in Figure 1B, the phosphorylation of calmodulin by the α-subunit can be also suppressed by a synthetic acidic peptide (β[55–71]) which encompasses a putative β-subunit down-regulatory domain (Boldyreff et al., 1992). This inhibitory effect of the 55–71 β-peptide is not merely due to competition at the substrate binding site, since it is absent if calmodulin is replaced by a variety of other substrates including the calmodulin-derived peptide RKMKDTDSEEEIR (Figure 1B), β-casein, and inhibitor 2 of protein phosphatase 1 (not shown), whose acidity is comparable to that of calmodulin. The latter finding also rules out the possibility that inhibitability by the β-subunit might be a feature of protein *vs* peptide substrates or a specific property of acidic protein substrates. On the other hand, the failure of the β-peptide to substitute for the β-subunit as a stimulator of CK2 basal activity monitored on the peptide substrate (compare panels A and B of Figure 1) is quite consistent with the knowledge that domains of the β-subunit distinct from the N-terminal acidic cluster of amino acids are essential for the positive interaction with the α-subunit (Boldyreff et al., 1993). In conclusion, the ability of the α-subunit to phosphorylate calmodulin can be specifically inhibited either by the β-subunit or, to a lesser extent, by a synthetic peptide that reproduces the β-subunit's 55–71 sequence.

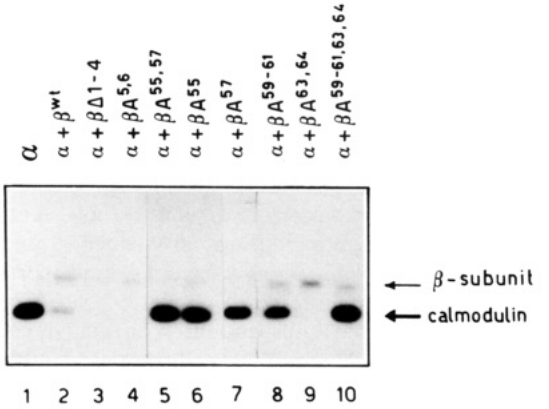

FIGURE 2: Inhibition of calmodulin phosphorylation by wild-type and mutated β-subunits. Calmodulin was phosphorylated by CK2 α-subunit either alone (lane 1) or combined with equimolar amounts of wild-type (lane 2) and mutated β-subunits (lanes 3–10). Experimental conditions are described under Materials and Methods. An autoradiogram of a dried SDS–PAGE gel is presented. For the nomenclature of the mutated β-subunits, see Table 1.

Effect of β-Subunit Mutations on Calmodulin Phosphorylation. In order to obtain additional information about the structural features of the β-subunit responsible for the inhibition of calmodulin phosphorylation, a variety of β-mutants were assayed for their ability to prevent calmodulin phosphorylation when added in equimolar amounts to the α-subunit. The results of a typical experiment run with a selection of representative mutants are shown in Figure 2. They clearly outline the failure of the mutant $\beta A^{55,57}$, but not of other mutants bearing substitutions at other positions or lacking the autophosphorylation ($\beta\Delta 1$–4) site, to prevent calmodulin phosphorylation. By use of the same approach, the ability of all the available mutants to affect the phosphorylation of calmodulin was evaluated and compared to that of the wild-type β-subunit. The calculated percentages of inhibition of calmodulin phosphorylation are given in Table 1. As expected, no inhibition was observed with those mutants with large C-terminal deletions, which are also unable to reconstitute the CK2 holoenzyme when added to the α-subunit in equimolar amounts (Boldyreff et al., 1993). Among the other mutants which were able to reconstitute active CK2 holoenzyme, only those bearing single, double, and triple substitutions in the triplet 55–57, or a quintuple substitution of acidic residues in the 59–64 sequence, have lost the ability to prevent calmodulin phosphorylation when combined in equimolar amounts with the α-subunit. Interestingly, these mutants are the same as those that give rise to a reconstituted CK2 holoenzyme displaying a basal activity toward canonical substrates that is higher than that of the wild-type holoenzyme (Boldyreff et al., 1993). Since the holoenzyme reconstituted with these mutants gives rise to a somewhat irregular sucrose gradient ultracentrifugation pattern exhibiting a shoulder of activity slightly more retarded than the main peak of heterotetrameric CK2, we monitored the activity toward calmodulin in both the shoulder and the main peak. As shown in Figure 3 for the holoenzyme reconstituted with mutant $\beta A^{55,57}$, the activity toward calmodulin is also predominant in the main peak as compared to the shoulder. This finding rules out the possibility that the residual calmodulin kinase activity observed with these mutants is due to impaired association with α-subunit, giving rise to oligomers defective in β-subunit. Altogether these data support a close correlation between hyperactivating properties of β-mutants and their inability to suppress calmodulin phosphorylation under basal conditions.

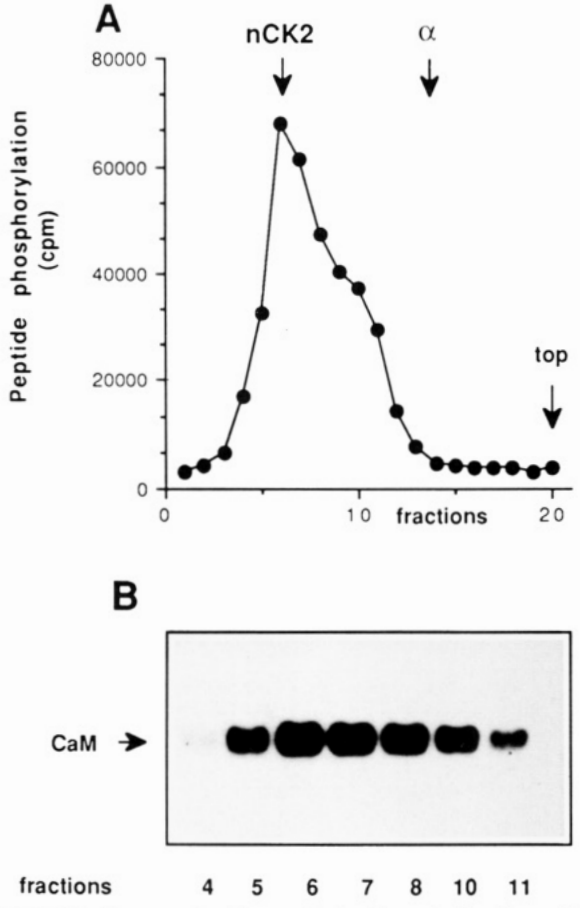

FIGURE 3: Sucrose density gradient ultracentrifugation of CK2 holoenzyme reconstituted with $\beta A^{55,57}$. Sucrose gradient ultracentrifugation was performed as described by Boldyreff et al. (1993). In panel A CK2 activity was monitored with the peptide substrate RRRDDDSDDD (100 μM). The positions of isolated α-subunit and native CK2 (nCK2) are indicated by arrows. In panel B individual fractions were assayed for their activity toward calmodulin as in Figure 2.

Effect of Polylysine on Calmodulin Phosphorylation by CK2 Reconstituted with Mutated β-Subunits. Since calmodulin is one of the CK2 substrates which is most sensitive to polylysine stimulation, it was of interest to check whether the structural features responsible for the down-regulation of calmodulin phosphorylation are also involved in the mechanism of polylysine activation. We therefore examined the responsiveness of variably reconstituted CK2 holoenzymes to polylysine concentrations sufficient to reverse the inhibition of calmodulin phosphorylation promoted by wild-type β-subunit. As shown in Table 2, the phosphorylation of calmodulin, catalyzed by the α-subunit, when inhibited by equimolar amounts of β^{wt}, can be fully restored by adding 42 nM polylysine. This results in an approximately 12-fold stimulation of the residual "calmodulin kinase" activity of the CK2 holoenzyme. A similar stimulation was observed with holoenzymes reconstituted with mutants $\beta\Delta 1$–4, $\beta\Delta 209$–215, and $\beta A^{5,6}$ (Table 2) and those mutants bearing substitutions in the 171–180 region (not shown). In contrast, no significant effect of polylysine was observed with holoenzymes reconstituted with the βA^{55-57}, $\beta A^{55,57}$, βA^{59-61}, and $\beta A^{59-61,63,64}$ mutants, i.e., those that proved the least effective in preventing calmodulin phosphorylation (see Figure 2 and Table 1). With increased polylysine concentration, the activity of CK2 holoenzyme reconstituted with mutant $\beta A^{55,57}$ is also increased (Figure 4A). The overall stimulation by polylysine, however, remains much lower than that observed with the wild-type reconstituted holoenzyme (Figure 4B).

Table 2: Effect of Polylysine on Calmodulin Phosphorylation by CK2 Holoenzyme Reconstituted with Wild-Type and Mutated β-subunits

subunit(s)	phosphorylation rate[a] (units/mg) –polyLys	phosphorylation rate[a] (units/mg) polyLys (42 nM)	average stimulation by polyLys (x-fold)
α	15.2 (±2.2)	16.4 (±2.1)	1.0
$\alpha + \beta^{wt}$	1.3 (±0.2)	15.6 (±1.7)	12.0
$\alpha + \beta^{55,57}$	10.3 (±2.2)	12.6 (±2.9)	1.2
$\alpha + \beta^{55-57}$	10.0 (±1.0)	8.0 (±0.9)	0.8
$\alpha + \beta A^{59-61}$	7.9 (±1.4)	12.6 (±2.8)	1.5
$\alpha + \beta A^{63,64}$	1.6 (±0.2)	11.2 (±2.4)	7.0
$\alpha + \beta A^{59-61,63,64}$	11.7 (±1.8)	12.9 (±2.0)	1.1
$\alpha + \beta\Delta 1$–4	1.3 (±0.2)	14.7 (±3.0)	11.3
$\alpha + \beta\Delta 209$–215	1.8 (±0.3)	14.7 (±1.5)	8.1
$\alpha + \beta A^{5,6}$	1.5 (±0.1)	11.5 (±1.9)	7.7

[a] Activity values are the mean of triplicate assays.

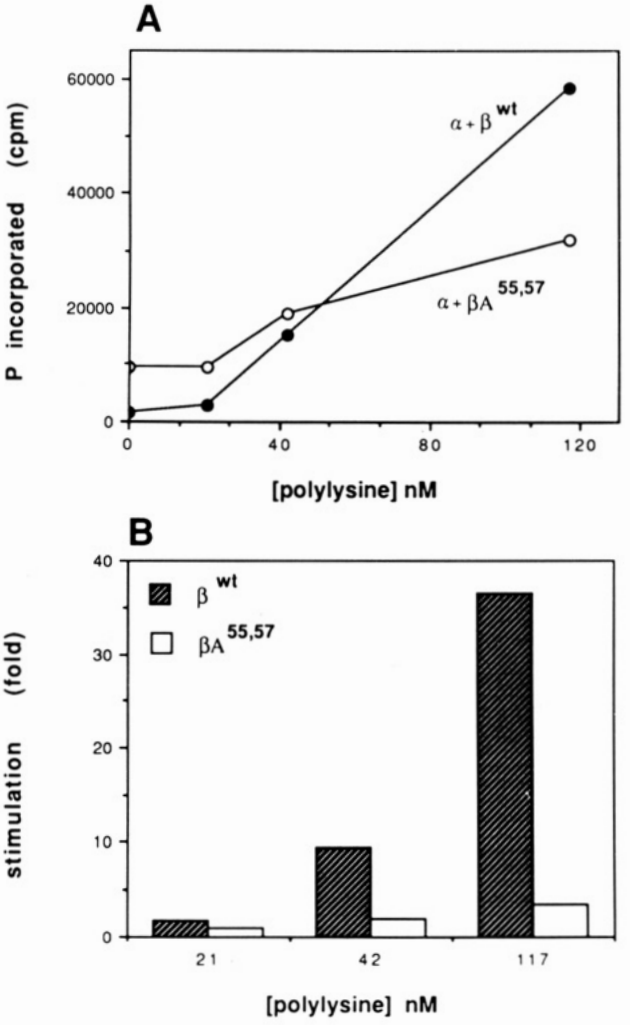

FIGURE 4: Polylysine-driven phosphorylation of calmodulin by CK2 holoenzymes reconstituted with either β^{wt} (●) or $\beta A^{55,57}$ (○). The effect of increasing polylysine concentration is shown in panel A. In panel B the stimulation by various polylysine concentrations relative to basal conditions (absence of polylysine) is reported. Phosphorylation was performed with 50 ng of α-subunit under conditions detailed under Materials and Methods.

The concept that residues Asp^{55} and Glu^{57} of the β-subunit play a relevant role in altering the catalytic activity of the α-subunit toward calmodulin and that this effect is relieved by polylysine is also consistent with the phosphoamino acid

Table 3: Phosphoamino Acid Analysis of Calmodulin Phosphorylated by Various Forms of CK2 with or without Polylysine[a]

enzyme	polyLys (42 nM)	SerP (cpm)	ThrP (cpm)	SerP/ThrP
α	–	12 700	15 515	0.81
	+	13 872	13 149	1.05
$\alpha + \beta^{wt}$	–	4702	2120	2.21
	+	36 930	39 672	0.94
$\alpha + \beta A^{55,57}$	–	16 794	13 479	1.24
	+	34 160	25 986	1.31

[a] Conditions for calmodulin phosphorylation and the isolation and evaluation of phosphoamino acids are detailed under Materials and Methods. The data are the average of three independent experiments. The standard error in all cases was less than 18% of the reported values.

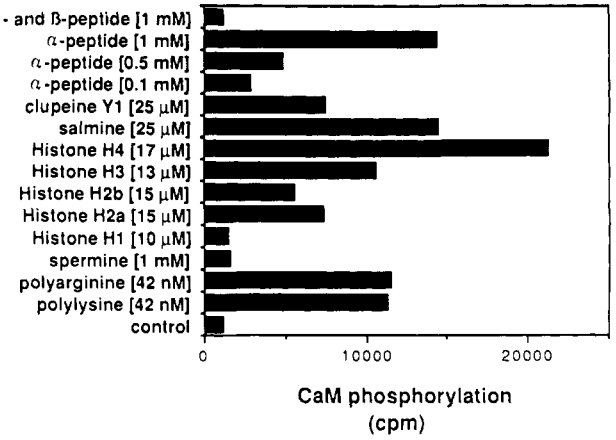

FIGURE 5: Phosphorylation of calmodulin by CK2 upon addition of various polycationic compounds. Phosphorylation of calmodulin by native CK2 holoenzyme either in the absence (control) or in the presence of various polycationic compounds (as indicated) was performed and evaluated as described under Materials and Methods. α-peptide and β-peptide denote the synthetic 66–86 and 55–71 fragments of CK2 α- and β-subunit, respectively.

analysis of phosphocalmodulin. As shown in Table 3, the β^{wt}-subunit not only dramatically reduces the phosphorylation of calmodulin by the α-subunit but also substantially increases the SerP/ThrP ratio of phosphocalmodulin. Polylysine, when added to the wild-type holoenzyme, restores both the catalytic activity toward calmodulin and a SerP/ThrP ratio similar to that observed using the free α-subunit as a catalyst. The SerP/ThrP ratio of calmodulin phosphorylated by the $\beta A^{55,57}$ holoenzyme is, in contrast, unaffected by polylysine and is like that found in calmodulin phosphorylated by the α-subunit.

Effectiveness of Various Polycationic Compounds in Triggering the Phosphorylation of Calmodulin. In the search for naturally occurring compounds that could act on the CK2 holoenzyme *in vivo* like polylysine *in vitro*, a number of polyamines and basic polypeptides were assayed for their ability to trigger the phosphorylation of calmodulin by the CK2 holoenzyme. A summary of the results is shown in Figure 5, where the effects of polylysine and polyarginine are also shown for reference. First, it should be noted that spermine, as well as the other polyamines spermidine and putrescine (not shown), which can stimulate CK2 activity under certain circumstances (Cochet & Chambaz, 1983; Hathaway & Traugh, 1984), is totally unable to promote calmodulin phosphorylation. A substantial calmodulin phosphorylation is triggered by histones H2a, H2b, and H3, and even more by H4, which could actually play a physiological role considering the high concentration of CK2 inside the nucleus (Filhol et al., 1990; Krek et al., 1992) and the existence of a nuclear pool of calmodulin (Bachs et al., 1992). In contrast, histone H1 is nearly ineffective, and under comparable conditions, the protamine clupeine Y1 is only half as effective as salmine, despite their high degree of homology (>75%). A remarkable activation of calmodulin phosphorylation by the CK2 holoenzyme can also be obtained upon addition of the basic peptide CVVKILKPVKKKKIKREIKILE, which reproduces the 66–86 insert of the CK2 α-subunit responsible for the binding of heparin (Hu & Rubin, 1990) and which has also been suggested to interact with the β-subunit (Lozeman et al., 1990; ole-MoiYoi et al., 1992). The activation by the α-peptide is indeed completely abolished by adding equimolar amounts of the β-peptide including the 55–64 acidic stretch (Figure 5), thus corroborating the view that the α- and β-peptide sequences recognize each other. It should be mentioned that several other basic peptides of similar size (e.g., KKKANLPKKEKSVLQGKLTKLA and KKDEKKKEKKNKK) proved either ineffective or much less effective than the α-peptide in triggering CK2 activity toward calmodulin (data not shown) consistent with the concept that the effect of polycationic peptides is quite specific and not merely due to an elevated number of positively charged residues.

DISCUSSION

All available data presented either here or in previous reports (Grankowski et al., 1992; Meggio et al., 1992a,b; Boldyreff et al., 1992, 1993) support the concept that the β-subunit of CK2 is needed to (re)constitute a fully active and stable enzyme but at the same time acts as a down-regulator of CK2 activity. Such a latter negative role of the β-subunit, detected by site-directed mutagenesis experiments (Boldyreff et al., 1992, 1993), is particularly evident if calmodulin is the substrate. Calmodulin is in fact an atypical substrate of CK2 inasmuch as its phosphorylation, as well as that of a few other substrates of CK2, is totally dependent on, instead of being merely stimulated by, polybasic peptides like polylysine. Such a behavior is especially notable since it is not intrinsic to the catalytic subunit of CK2, which readily phosphorylates calmodulin in the absence of any effector (Meggio et al., 1992b; Bidwai et al., 1993), but is imposed by the noncatalytic β-subunit of CK2, which assembles with the α-subunit to give heterotetramers ($\alpha_2\beta_2$). The resulting holoenzyme exhibits an overall increased activity toward most substrates (Cochet & Chambaz, 1983; Grankowski et al., 1991; Filhol et al., 1991; Birnbaum et al., 1992), while it is nearly inactive toward calmodulin unless polybasic effectors are added (Meggio et al., 1992b; Bidwai et al., 1993). These properties render calmodulin the first choice as substrate for investigating the molecular mechanism by which CK2 could undergo regulation. The physiological relevance of this regulation is supported by the finding that calmodulin is phosphorylated *in vivo* at the same residues phosphorylated *in vitro* by CK2 (Nakajo et al., 1986; Sacks et al., 1992).

This paper provides evidence that a series of acidic residues clustered in the N-terminal moiety of the CK2 β-subunit are responsible for an intrinsic down-regulation of the CK2 holoenzyme, causing an almost complete inhibition of calmodulin phosphorylation, which can be relieved by a variety of basic polypeptides but not by polyamines. The following evidence supports this conclusion: (1) Single and multiple substitutions of Ala for the acidic residues located in the 55–64 segment give rise to mutants that, when added in equimolar amounts to the recombinant α-subunit, reconstitute holoenzymes whose activities are up to 8-fold higher than that of the

holoenzyme reconstituted with the wild-type β-subunit (Boldyreff et al., 1992, 1993). (2) While the reconstitution of wild-type holoenzyme ($\alpha_2\beta_2$) causes a complete loss of CK2 catalytic activity toward calmodulin, only a partial to neglegible loss of calmodulin kinase activity is observed if the holoenzyme is reconstituted with mutants having Ala variably substituted for Asp/Glu residues in the 55–64 sequence. (3) A specific decrease of phosphorylating activity toward calmodulin, but not toward other substrates, is also induced by adding to the α-subunit a synthetic peptide encompassing the 55–71 sequence of the β-subunit and including the crucial acidic residues. (4) Once the calmodulin kinase activity of the α-subunit has been suppressed by assembly with equimolar amounts of wild-type β-subunit, it can be recovered by the addition of polylysine at concentrations that are ineffective with either the α-subunit or the holoenzymes reconstituted with β-subunits mutated at the 55–57 sequence. (5) Other polycations can replace polylysine with variable effectiveness: e.g., polyarginine is as effective as polylysine; histones H2a, H2b, H4, and H3 also restore the calmodulin kinase activity of the CK2 holoenzyme, while histone H1 is nearly ineffective, and polyamines, under our experimental conditions, proved totally ineffective. (6) A synthetic peptide, CVVKILKPVKKKKIKREIKILE, encompassing the basic 66–86 insert of the α-subunit (but not other peptides of similar size and basicity) can also trigger calmodulin phosphorylation by CK2 holoenzyme, its effect being fully abolished by the addition of equimolar amounts of a synthetic peptide encompassing the 55–71 sequence of β-subunit.

These observations, besides highlighting a region of the β-subunit which is crucial for the down-regulation of CK2-catalyzed calmodulin phosphorylation and for the response to polybasic effectors, are consistent with the view that a polycationic region of the α-subunit, required for full catalytic activity, is engaged, under basal conditions, with the acidic residues clustered in the N-terminal moiety of the β-subunit, thus resulting in a negative regulation of activity. Such an "autoinhibition" could be relieved by polycationic effectors that by virtue of their structural similarity to the α-subunit basic segment outcompete it from the acidic stretch of the β-subunit. It should be noted in this connection that the basic α[66–86] peptide does not prevent the association of the α- and β-subunits to reconstitute the holoenzyme as judged by sucrose gradient ultracentrifugation experiments. This is consistent with the notion that the 55–64 acidic cluster of the β-subunit responsible for down-regulation is not required for assembly of the α- and β-subunits, which is rather dependent on the integrity of the β-subunit C-terminal domain (Boldyreff et al., 1993). The final identification of the basic 66–86 insert as the regulatory element which interacts with the 55–64 segment of the β-subunit will need further experiments. It is consistent, however, with all the data presented here as well as with the finding that this insert mediates the effect of heparin (Hu & Rubin, 1990), one of the most powerful and specific inhibitors of CK2 (Hathaway et al., 1980). The wild-type β-subunit protects the catalytic subunit against heparin inhibition more effectively than the mutants $\beta A^{55,57}$ and βA^{55-57} (Boldyreff et al., 1993).

This mechanism of regulation raises the question of what the physiological effectors/events are that are mimicked *in vitro* by polybasic peptides. While an answer could be only conjectural at this stage, it should be noted that the effects displayed *in vitro* by large polycations like polylysine are multifarious and are not entirely explicable by the interactions of the polycations with the 55–64 acidic cluster of the β-subunit. This interaction appears to play a primary role in relieving the inhibition of the calmodulin kinase activity imposed by the β-subunit over the catalytic subunit. It hardly accounts, however, for the extra stimulation of activity observed on increasing the polylysine concentration, using either calmodulin or "canonical" substrates. It is obviously unrelated to the low but significant responsiveness to polylysine displayed by the α-subunit alone (Meggio et al., 1992b). On the other hand, it is conceivable that a large artificial molecule like polylysine may display an array of effects, some of which may be physiologically significant, e.g., triggering latent activity toward endogenous substrates, while others could merely represent *in vitro* artifacts.

It is worthy of note in this respect that the α-peptide is more selective than polylysine as an inducer of CK2 activity toward calmodulin, especially since, at concentrations capable of triggering calmodulin phosphorylation, it does not display some of the side effects of polylysine such as the autophosphorylation of CK2 at its catalytic subunit (Meggio et al., 1983) and the stimulation of the kinase activity of the isolated α-subunit (Meggio et al., 1992b) (unpublished data). Additional studies with α-peptide derivatives bearing suitable modifications could disclose the structural features that are specifically capable of eliciting the latent activity toward calmodulin and toward other physiological targets of CK2 whose phosphorylation is almost undetectable under basal conditions, e.g., ornithine decarboxylase (Meggio et al., 1984), clathrin β light chain (Bar-Zvi & Branton, 1986), and elongation factor 1 (Palen et al., 1990). It has to be assumed that the *in vivo* phosphorylation of these substrates is crucially dependent on a mechanism of CK2 activation by polycationic effectors, like the one outlined in this study.

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