Activity of Recombinant α and β Subunits of Casein Kinase II from *Xenopus laevis*†

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ABSTRACT: Casein kinase II (CKII) is a ubiquitous protein kinase, found predominantly in cell nuclei, which has two subunits in a tetrameric $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ conformation. The catalytic center is present in the α subunit which is active by itself while β is a regulatory subunit that can greatly enhance the activity of α. The cDNA genes of *Xenopus laevis* coding for the α and β subunits of CKII have been expressed in *Escherichia coli* and extensively purified. The recombinant subunits reconstitute a fully active holoenzyme when incubated in stoichiometric amounts. Mutations that change serines in positions 2 and 3 of the β subunit for glycines completely eliminate the autophosphorylation site present in this subunit but do not significantly affect the capacity of β to activate α. A fusion protein composed of glutathione transferase linked to the *X. laevis* CKII β subunit can also activate α. This fusion protein binds to glutathione–agarose beads and can mediate the binding of the α subunit to this matrix. Conversely, the α subunit was found to bind to glass fiber filters in an active form that can still be activated by β to an extent similar to that seen in solution. Using peptides containing tyrosine and glutamic acid as inhibitors of the activity of the isolated α subunit and of the holoenzyme, the effect of β on the specificity of inhibition was studied. The results obtained demonstrate that the β subunit participates in the specific recognition of tyrosine by the holoenzyme and in the discrimination in the position of the tyrosyl residues with respect fo the acidic amino acids.

Casein kinase II (CKII) is a protein kinase that is present in all eukaryotic cells and is predominantly localized in the cell nucleus [reviewed in Pinna (1990) and Tuazon and Traugh (1991)]. This enzyme has been found to be a tetramer of an $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ conformation. The α or α' subunits contain the catalytic center and are active by themselves while β is a regulatory subunit that greatly stimulates the activity of α (Cochet & Chambaz, 1983).

CKII phosphorylates serines or threonines that are present in acidic regions of proteins. The study of sequences phosphorylated by CKII and the use of model peptides has defined a minimum recognized substrate sequence with the structure Ser/Thr·X·X· acidic residue. The acidic residue can be an aspartic acid, glutamic acid, or phosphorylated serine, threonine, or tyrosine. The X residues are commonly also acidic in nature (Meggio et al., 1984; Kuenzel & Krebs, 1985).

The physiological mechanism for the regulation of CKII activity is not known; however, there are several reports that indicate that its activity varies in cells stimulated to divide by various mitogenic stimuli (Klarlund & Czech, 1988; Ackerman & Osheroff, 1989). *In vitro*, it has been found that CKII activity can be significantly stimulated by polyamines such as spermine and spermidine and by polylysine (Cochet et al., 1980; Meggio et al., 1987).

Heparin has been found to be a very potent inhibitor (Hathaway et al., 1980); however, the strength of the inhibition depends on the nature of the substrate protein (Taylor et al., 1987). Other polyanions such as polyglutamic and polyaspartic acid and some nucleic acids have also been found to be inhibitory (Meggio et al., 1983; Gatica et al., 1989). Interestingly, the presence of tyrosine in acidic clusters greatly enhances the inhibitory capacity of these polymers (Meggio & Pinna, 1984; Tellez et al., 1990).

CKII has been found to phosphorylate a large number of different proteins both *in vivo* and *in vitro*. Special importance has been given to the fact that CKII phosphorylates several enzymes and factors involved in the synthesis of nucleic acids and in the regulation of transcription. In the case of topoisomerase II (Corbett et al., 1992) and the protein products of the protooncogenes myb (Lüscher et al., 1990), jun (Lin et al., 1992), and max (Berberich & Cole, 1992), it has been reported that CKII phosphorylation modifies the physiological activity of these proteins.

The cDNAs coding for the α, α', and β subunits of CKII from humans, drosophila, yeast, and several other species have been cloned and sequenced (Saxena et al., 1987; Meisner et al., 1989; Grankowski et al., 1991; Hu & Rubin, 1990a). In our laboratory, we have reported the cloning and sequence of the α and β subunits of the *Xenopus laevis* CKII (Jedlicki et al., 1992). Analysis of these sequences shows a remarkable degree of conservation in evolutionarily distant species; for instance, the sequences from *Xenopus* and humans of the α subunit differ only in 1 amino acid in the first 300 amino acids, and the 2 β subunits of the same species differ only in 2 amino acids over the whole length of the polypeptide.

Expression and mutagenesis of cloned subunits of CKII are presently being used to determine the structure–function relationship in the mechanism of action of this enzyme. There have been studies that indicate that the β subunit, in addition to its stimulatory capacity, also influences the specificity in the interaction of the enzyme with its substrates (Meggio et

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al., 1992) or with metal ions (Gatica et al., 1993). Deletions and mutations of the β subunit have been used to determine that there are regions of the molecule that are dispensable for its stimulatory activity and other regions that can significantly affect this capacity (Boldyreff et al., 1992). Likewise, it has been determined that mutations of the α subunit can alter its capacity to be inhibited by heparin (Hu & Rubin, 1990b) or its response to β activation (Jakobi and Traugh, 1992).

The present report describes the successful expression in *Escherichia coli* of the recombinant cDNA genes coding for the α and β subunits from *Xenopus laevis*. The resulting proteins have also been obtained in highly purified form. The availability of these recombinant subunits has enabled us to conduct studies on the interaction of the two isolated subunits and on the effects of this interaction on the function of the enzyme. It has been found that the recombinant α and β subunits can spontaneously interact to reconstitute the holoenzyme. Mutations that eliminate the autophosphorylation sequence from the β subunit do not significantly affect the capacity of β to activate α.

Fusion of the β-polypeptide with glutathione transferase yields a fusion protein that can still bind and activate α and can serve to bind the enzyme to a solid matrix of glutathione–agarose beads. Conversely, the α subunit can be immobilized in an active form on glass fiber filters and can still be activated by β on this solid matrix.

The use of peptides containing tyrosine and glutamic acid residues as inhibitors of CKII activity demonstrates that the β subunit plays an important role in the recognition of the tyrosine residues in these inhibitory peptides.

MATERIALS AND METHODS

Materials. Human thrombin, glutathione–agarose beads, and the amino acid polymers poly(glutamic acid) (M_r 43 000), random copoly(Glu:Tyr) (4:1) (M_r 46 000), and random copoly(Glu:Phe) (4:1) (M_r 16 000) were purchased from Sigma Chemical Co.

Restriction enzymes were purchased from Promega. Expression vector pT7-7 was obtained from Dr. Juan Olate and pGEX-2T from Dr. Silvio Gutkind.

Preparation of CKII from Xenopous laevis Oocyte Nuclei. Ovaries were obtained by surgery of adult female *X. laevis*. State 5 and 6 oocyte nuclei were prepared as described by Burzio and Koide (1976). Highly purified CKII was prepared from isolated oocyte nuclei by chromatography on DEAE-Sephadex and phosphocellulose (Taylor et al., 1987; Hathaway & Traugh, 1983). The specific activity of an average preparation of the holoenzyme was 25 units/mg (1 unit is the amount of enzyme transferring 1 nmol of phosphate to casein per minute at 30 °C under standard assay conditions).

Assays of CKII Activity. Reactions (50 μL) contained 50 mM Hepes, pH 7.8, 150 mM KCl, 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (500–1000 cpm/pmol). Assays also contained CKII holoenzyme or recombinant α subunit and 5.0 mg/mL dephosphocasein. The reaction was started by the addition of the enzyme. Incubations were 10 min at 30 °C. It has been determined that under these conditions the reaction is linear for at least 30 min. An aliquot was spotted on a 2 × 1 cm Whatman P81 phosphocellulose paper which was then immersed in 75 mM phosphoric acid. The paper was washed 3 times in the same acid, dried, and counted. Values reported have been corrected for controls run in the presence of heat-denatured enzyme. All assays were performed in duplicate and are representative of two to four experiments.

Expression and Purification of the α and β Subunits of CKII. Cloning and sequencing of the CKII α and β subunits were described earlier (Jedlicki et al., 1992). A clone containing a 2000 bp insert with 1050 bp corresponding to the α subunit coding region was amplified by PCR using oligonucleotide primers containing *Pst*I restriction sites complementary to the sequence bordering the coding region. The sequence of the upstream primer was 5′CCCCTGCAGATGTCAGGACCTGTG3′ and of the downstream primer 5′CCCCTGCAGTCATACTGGCGCTACT3′. The amplified and *Pst*I-digested fragment was subcloned into the expression vector PT7-7 (Tabor & Richardson, 1985) and introduced into the *E. coli* expression strain BL21(DE3). Expression was induced with 1 mM isopropyl β-D-thiogalactoside. Cells were harvested after 2-h incubation and resuspended in 20 mM Tris-HCl, pH 7.5, buffer containing 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL leupeptin, and 0.7 μg/mL pepstatin. Cells were sonicated and centrifuged at 12000*g* for 30 min. The α subunit of CKII present in the supernatant solution was purified on DEAE-Sephadex and phosphocellulose as described for the holoenzyme (Hathaway & Traugh, 1983). The average yield of α subunit was 3 mg of protein/L of bacterial culture. The protein was stored in 25 mM potassium phosphate buffer, pH 7.5, in 0.7 M KCl at 4 °C. The specific activity obtained in different preparations ranged from 65 to 90 units/mg, assayed under standard conditions described above for the holoenzyme.

The expression of the β subunit was achieved by use of a pGEX vector to produce a fusion protein with glutathione *S*-transferase (Smith & Johnson, 1988). A clone containing the entire coding region of the CKII β subunit was subcloned in the *Eco*RI site of the expression vector pGEX-2T after introducing *Eco*RI sites in both extremes of the β-coding region and amplification by PCR using synthetic oligonucleotide primers. The sequence of the upstream primer was 5′TACGAATTCAAAATGAGTAGCTCG3′ and the downstream primer 5′GGGGAATTCTCAACGCATGGTCT3′. The ligation product was used to transform *E. coli* AG1, and transformants were selected on LB/ampicillin plates. The fusion protein was purified on glutathione–agarose beads exactly as described (Smith & Johnson, 1988). Removal of the glutathione transferase moiety was achieved by incubation with 30 μg of human thrombin in 2.5 mM $CaCl_2$ for 1 h at 30 °C. The glutathione transferase was separated from the β subunit by a second passage of dialyzed fusion protein through the glutathione–agarose column. The purified β subunit was stored in 50 mM Tris-HCl, pH 7.5, in 150 mM NaCl at 4 °C.

Mutation of the Autophosphorylation Site of Subunit β. Serines in positions 2 and 3 of the β subunit were mutated through PCR amplification of the cloned cDNA gene (Jedlicki et al., 1992). The appropriate base changes were introduced in the upstream primer which had the sequence 5′CCCGAATTCAAATGGGTGGCTCGGA3′ (the underlined bases are the ones changed from the wild-type sequence). The downstream primer was the same as described previously for cloning into the pGEX-2T expression vector. The amplified DNA was again introduced into that vector, and an identical procedure was followed to express and purifiy the mutated β subunit. The entire nucleotide sequence of the mutated expression clone was corroborated by sequencing (Sanger et al., 1977).

Analysis of the Association of CKIIα with CKIIβ Fusion Protein. The fusion protein glutathione transferase–β subunit

(50–60 μg in a typical experiment) obtained as described above was incubated 15 min at room temperature with 100 μL of a 50% slurry of GSH–agarose in a 200-μL final volume. The GSH–agarose was washed by microcentrifugation (the supernatant was designated the S-1 fraction) with 5 × 1 mL of PBS. Subunit α (45 μg) was added to the washed agarose containing the bound fusion protein in a final volume of 0.8 mL in 20 mM potassium phosphate buffer, pH 6.8, and 0.5 M NaCl. Incubation of α subunit with the bound fusion protein was for 1 h at 4 °C with gentle mixing. The slurry was microcentrifuged for 15 s, and the supernatant solution (designated the S-2 fraction) was assayed for kinase activity and analyzed by 10% SDS–polyacrylamide gel electrophoresis. The agarose beads were then washed by centrifugation with 5 × 1 mL of PBS and resuspended in 300 μL of PBS. Aliquots of the slurry were then used for kinase assays, and for gel analysis after the addition of a 2× SDS gel sample buffer.

Adsorption of α to Glass Fiber Filters. The retention of α, or α and β, subunits of CKII on glass fiber filters was as follows: α or α plus β subunits containing between 0.5 and 10 μg of protein in 100 μL of 25 mM Hepes, pH 7.5, and 0.7 M KCl were incubated with an 0.8 cm^2 GF/C glass fiber filter for 15 min at 4 °C. The filters were washed free of unbound enzyme by incubating 3 times consequently in 100 mL of 25 mM Tris-HCl, pH 7.5, at 4 °C for 5 min. Each filter was blotted quickly after Whatman no. 3 filter paper and transferred to a 1.5-mL Eppendorf tube for protein kinase assay. The standard protein kinase assay with casein as substrate was used except with a final volume of 100 μL. An aliquot of 60 μL was removed for measurement of [^{32}P]-phosphate incorporation. Controls were performed using filters without protein or filters with α or α + β bound subunits subjected to 100 °C for 3 min prior to assay. In assays using 1.5 × 10^6 cpm, these blanks yielded less than 1500 cpm for both boiled filters and filters not exposed to enzyme. The incorporation of [^{32}P]phosphate is proportional to the amount of enzyme protein used during the adsorption step.

The possibility that α subunit was released from the filter during the assay period was tested by the incubation of 8.5 μg of α subunit bound to a filter as described above but without substrates. After centrifugation at 12 000 rpm for 3 min, the supernatant solution was found not to contain kinase activity.

Microsequencing of Recombinant β Subunit. Amino-terminal amino acid sequencing of the CKII β subunit produced after thrombin treatment of the fusion protein was performed by the Peptide and Protein Service LANAIS-PRO, Buenos Aires, Argentina, and the sequence GSPGIQM... was determined. The first six amino acids of the amino terminal originate from the fusion protein.

Peptide Synthesis. The peptides YYEEEEEEEEE (Y_2E_9) and EEEEEEEEYY (E_9Y_2) were synthesized using a commercial peptide synthesizer (Model 950 Pep Synthesizer, Milligen/Bioresearch). Their purity was checked by HPLC, and their composition was corroborated by amino acid analysis.

Oligonucleotide Synthesis. Oligonucleotides for PCR primers and for sequencing reactions were synthesized in an Applied Biosystems DNA synthesizer, Model 381A.

Other Procedures. Protein determinations were performed by the method of Bradford (1976) using bovine serum albumin as standard. SDS–polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (1970). Autoradiography was performed using Kodak X-Omat AR film.

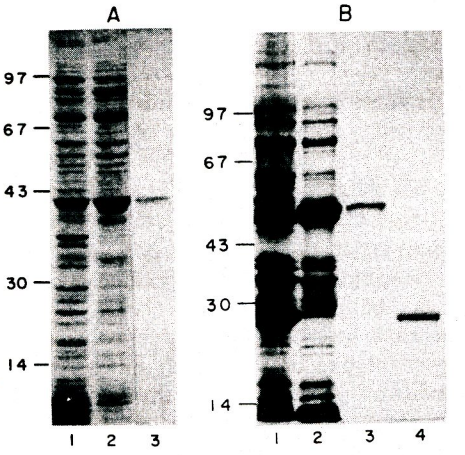

FIGURE 1: Steps for the purification of CKII α and β subunits expressed in *E. Coli*. Details of procedures are presented under *Materials and Methods*. (Part A) Coomassie Blue stained 10% polyacrylamide gel analysis of protein fractions: lane 1, extract of the *E. coli* strain BL21(DE3) transformed with the pT7-7 vector with the α cDNA gene and induced with IPTG; lane 2, CKII activity obtained after DEAE-Sephadex; lane 3, CKII fraction after phosphocellulose chromatography. (Part B) Coomassie Blue stained 10% polyacrylamide gel analysis of the fractions used to obtain purified recombinant β subunit: lane 1, extract of a control culture of AG-1 *E. coli* transformed with pGEX-2T without insert and induced with IPGT; lane 2, a similar extract but in which pGEX-2T has the cDNA for the β subunit inserted to produce a fusion protein of M_r 52 000; lane 3, analysis of the fusion protein obtained by affinity chromatography on GSH–agarose; lane 4, β subunit obtained after thrombin digestion and rechromatography through GSH–agarose.

RESULTS

Expression and Purification of the Recombinant α and β Subunits of CKII from X. laevis. The cloned cDNA gene coding for the α subunit of *Xenopus laevis* CKII was introduced into a PT7-7 expression vector and expressed in *E. coli* as detailed under Materials and Methods. Purification of the *X. laevis* α subunit from the bacterial extracts by conventional column chromatography on DEAE-Sephadex and phosphocellulose yielded a highly purified enzyme preparation with a main electrophoretic band of approximately M_r 42 000 (Figure 1A). This enzyme had a specific activity of 65 units/mg of protein. This specific activity is similar to that of other purified preparations of CKII α subunits reported in the literature (Grankowski et al., 1991; Lin et al., 1991).

Likewise, the cDNA gene coding for the β subunit of *X. laevis* was expressed in *E. coli* as a fusion protein using a pGEX-2T expression vector. The results of the purification on GSH–agarose and cleavage of this fusion protein (M_r 52 000) by thrombin are shown in Figure 1B. The β subunit hydrolyzed from the fusion protein (M_r 26) has six additional amino acids at its amino-terminal end with the sequence $GSPGIQM^1S^2S^3S^4E^5E^6$... (the amino acids numbered belong to the normal β sequence). The correctness of the predicted sequence and the purity of this subunit preparation was verified by microsequencing the first seven amino acids of the amino-terminal end. The expression and purification procedure yielded approximately 2 mg of β subunit per liter of culture of the transformed *E. coli*.

Stimulation of α by Wild-Type β and by a Mutant β Lacking the Autophosphorylation Site. Addition of wild-type β subunit to the purified recombinant α increases its catalytic activity 5-fold. As seen in Figure 2, stoichiometric amounts of β are required to yield maximum activation of α.

It has long been known that the β subunit of CKII is autophosphorylated (Hathaway & Traugh, 1979). Recent

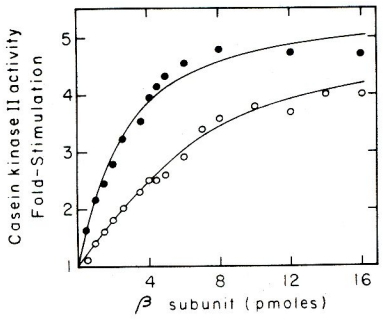

FIGURE 2: Activation of the α subunit of CKII by subunit β. Activation of CKII α-subunit activity was assayed as described under Materials and Methods, using 8 pmol of the α subunit and varying amounts of β subunit. The basal activity without β (19 pmol/min) has been subtracted from all values. Data analysis was performed using a program based on the algorithm of Marquardt (1963) for nonlinear fitting. The wild-type recombinant β (●) or the mutant β (○), in which serines in positions 2 and 3 have been replaced by glycines, was used.

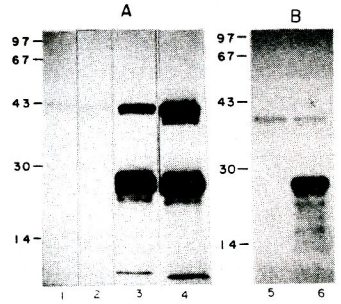

FIGURE 3: Autophosphorylation of the CKII α and β subunits and the effect of polylysine. The α (M_r 42 000) and β (M_r 26 000) subunits of CKII that had been incubated with [γ-^{32}P]ATP in the standard assay mixture but in the absence of casein were analyzed by autoradiography on 10% SDS–polyacraylamide gels; 40 pmol of α subunit or of both α and β subunits were used. (A) Incubations contained α subunit (lanes 1 and 2) or α and β subunits (lanes 3 and 4); without additions (lanes 1 and 3) or with 40 μg/mL polylysine (lanes 2 and 4). (B) Incubations contained α subunit and either the mutant β with glycines in positions 2 and 3 (lane 5) or the wild-type β subunit (lane 6). The autoradiography shown in (A) was exposed for 28 h while that for (B) was exposed for 12 h.

work from the group of Krebs (Lichfield et al., 1991) has established that the autophosphorylation site resides in the sequence MS*S*SEE which is at the amino-terminal end of β. In this sequence, serines-2 and -3 (with asterisks) fit the minimum requirements for CKII phosphorylation. Using PCR mutagenesis, a mutant β-subunit cDNA has been prepared in which the triplets coding for these two serines have been changed to code for glycines. The mutant β subunit expressed and purified exactly as the wild-type β was tested for its capacity to activate α. The results obtained are shown in Figure 2 and demonstrate that the mutant β is capable of activating α to an extent similar to the wild-type subunit. Variations in the degree of the stimulatory capacity of different preparations of β were between 4- and 5.5-fold activation.

The results presented in Figure 3 are autoradiographs of the CKII subunits incubated with [γ-^{32}P]ATP and subsequently analyzed on SDS–polyacrylamide gels. Figure 3A demonstrates the pronounced phosphorylation of β (M_r 26) by the α subunit. It can also be seen that the α subunit (M_r 42) is also slightly autophosphorylated and that this phosphorylation is stimulated by the presence of β. Polylysine, a known activator of CKII activity, which apparently operates through β, does not stimulate the autophosphoryation of α when incubated in the absence of β but stimulates moderately the autophosphorylation of β and of α when β is present. In

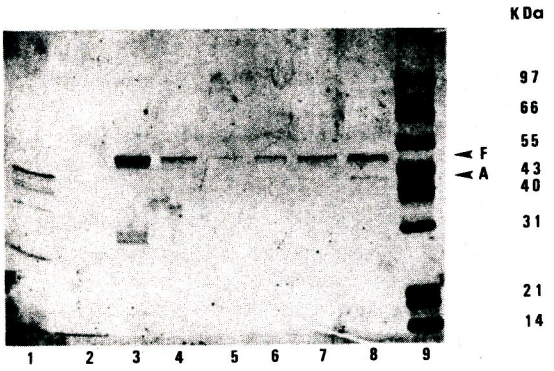

FIGURE 4: Binding of the α subunits to GSH–agarose beads in the presence of glutathione transferase–β-subunit fusion protein. CKII α subunit was incubated with GSH–agarose beads previously treated to contain bound fusion protein glutathione transferase–β subunit as described under Material and Methods. After being washed and resuspended in a 300-μL volume, the proteins present in aliquots of the bead slurry were analyzed along with control fractions on 10% polyacrylamide gels and protein stained with Coomassie Blue. Lane 1, 10 μL (2.8 μg) of α subunit before incubation with GSH–agarose-bound fusion protein. Lane 2, 50 μL of the supernatant after incubation of α with the fusion protein bound to the beads (S-2 fraction). Lane 3, 10 μL (5.4 μg) of fusion protein before incubation with GSH–agarose. Lane 4, 10 μL of the supernatant of GSH–agarose after incubation with fusion protein (S-1 fraction). Lanes 5–8, 5, 10, 20, and 50 μL of GSH–agarose slurry containing bound fusion protein, after incubation with α subunit. Lane 9, migration of protein markers. The arrowheads indicate the migration of fusion protein (F) and α subunit (A).

a separate experiment presented in Figure 3B, it is observed that the Gly^2-Gly^3 mutant of β is not phosphorylated by α, confirming the finding that Ser^2 and Ser^3 are the only autophosphorylation sites of this subunit.

Interaction of Subunits Attached to a Solid Matrix. Studies of protein–protein interaction can be facilitated by the attachment of one of the protein components to a solid matrix.

One of the byproducts of the expression of the β subunit is the fusion protein containing glutathione transferase in its amino-terminal half and the CKII β subunit in its carboxy-terminal end. This fusion protein can interact in solution with α as shown by the fact that it can stimulate its activity almost as much as the free β subunit resulting from thrombin treatment of the fusion chimera. For instance, in experiments in which free β yielded a 4.4-fold stimulation of α, an equimolar amount of fusion protein yielded a 3.2-fold stimulation.

The fusion protein has the useful property of binding with high affinity and specificity to glutathione–Sepharose beads. The possibility was explored, therefore, of using the fusion protein to mediate the attachment of the catalytically active α to these beads. The binding of CKIIα subunit to GSH–agarose–fusion protein is confirmed by detection of the 42-kDa protein together with the fusion protein of 52 kDa upon polyacrylamide gel analysis of the GSH–agarose preparation. These bands are observed despite the thorough washing of the GSH–agarose beads in buffered saline. Figure 4 (lanes 5–8) shows the increment in the 42-kDa protein band obtained upon analysis of increasing amounts of the GSH–agarose preparation. Parallel analysis of aliquots of the same preparation showed proportional amounts of CKII activity; for example, 5 and 10 μL of the bead slurry (as used in Figure 4, lanes 5 and 6) had an activity of 5 and 12 pmol/min, respectively.

The supernatant obtained after incubation of the α subunit and the GSH–agarose fusion protein (S-2 fraction) contained less than 10% of the total kinase activity used, and no protein band was detectable upon gel analysis (lane 2).

Table I: Activity of the α Subunit of CKII Immobilized on Glass Fiber Filters[a]

expt	amount of subunit(s) α or α + β used for immobilization (μg) α	β	additions	filter act. (pmol of ^{32}P/min)
1	0.85			0.8
	4.25			2.2
	8.5			5.4
2	8.5			3.0
	8.5		heparin (10 μg/mL)	0.4
3	8.5			3.5
	8.5		β subunit (10 μg)	17.0
4	8.5	10		16.0
	8.5	10	heparin (10 μg/mL)	0.7

[a] The experimental procedures and assay are described under Materials and Methods.

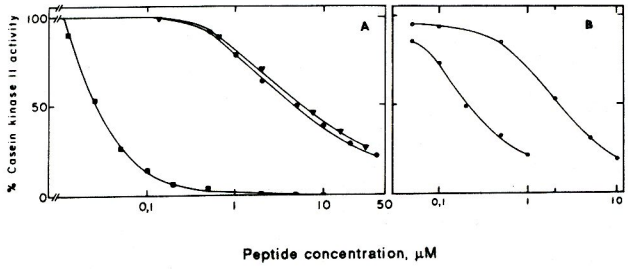

FIGURE 5: Comparison of the inhibition of CKII holoenzyme and recombinant α subunit by polypeptides rich in glutamic acid. (A) Assays were performed as described under Materials and Methods, using 2.4 milliunits of CKII holoenzyme, purified from *X. laevis* oocyte nuclei, in the presence of the sodium salts of poly(glutamic acid) (M_r 43 000) (●), copoly(glutamic:phenylalanine) (4:1) (M_r 16 000) (▲), or copoly(glutamic:tyrosine) (4:1). (M_r 46 000) (■). (B) Assays were performed as described under Materials and Methods except with 120 mM KCl and with 3.7 milliunits of recombinant α subunit in the presence of varying concentrations of poly(glutamic acid) (○) or copoly(glutamic:tyrosine) (4:1) (●). 100% corresponds to the activity in the absence of inhibitor.

Conversely, it has been observed that the α subunit can bind tightly to glass fiber filters. As shown in Table I, the CKII activity bound to these filters increases proportionately with the concentration of the α subunit originally incubated with the filters.

It is also apparent from this table that the holoenzyme is also tightly bound to the glass fiber matrix and that both the immobilized holoenzyme and α subunit retain the property of being strongly inhibited by heparin. The most important observation presented in this table, however, is the stimulation of the bound α by β subunit. The activation observed is at least as high as that measured with α in solution. Separate experiments have shown that the capacity of β to activate α does not depend on the original concentration of the immobilized α. The activity of the α subunit bound to glass fiber is labile at 37 °C but can be greatly stabilized by the addition of β subunit.

Influence of the β Subunit in the Recognition of Peptide Inhibitors Containing Tyrosine and Glutamic Acid. It has been shown previously that the CKII holoenzyme can be strongly inhibited by random copolymers of glutamic acid and tyrosine (Meggio & Pinna, 1989; Tellez et al., 1990). In Figure 5A, it can be observed that copoly(Glu:Tyr) (4:1) is more than 2 orders of magnitude more potent than poly(glutamic acid) or copoly(Glu:Phe) (4:1) in inhibiting the holoenzyme. A similar experiment carried out with the isolated recombinant α subunit is shown in Figure 5B. Although copoly(Glu:Tyr) (4:1) is still considerably stronger than poly(glutamic acid), the difference between the potency of the two inhibitors is reduced to 1 order of magnitude.

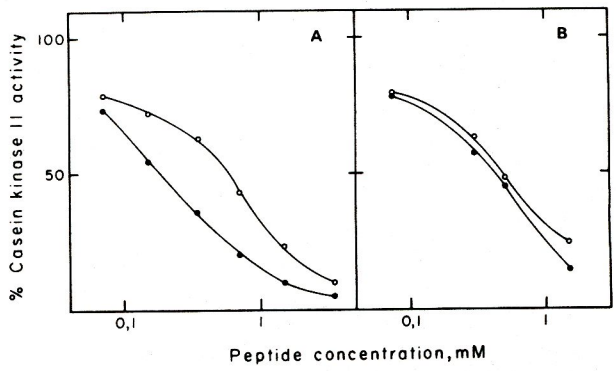

FIGURE 6: Comparison of the inhibition of CKII holoenzyme and recombinant α subunit by acidic synthetic peptides containing tyrosine. Assays were performed as described under Materials and Methods except that in (B) 120 mM KCl was used. (A) CKII holoenzyme (2.9 milliunits) or (B) recombinant α subunit (3.7 milliunits) was assayed in the presence of varying concentrations of the peptides E_9Y_2 (●) and Y_2E_9 (○). 100% activity is the activity in the absence of peptide inhibitor.

Using synthetic undecapeptides containing two tyrosines and nine glutamic acids, the importance of the position of the tyrosines with respect to the polyglutamic cluster was tested.

Figure 6A shows the inhibitory capacity of two such peptides, containing the tyrosines either in the amino end or in the carboxy end, toward the activity of the CKII holoenzyme. It is evident that the Y_2E_9 peptide is a significantly better inhibitor than the one with the sequence E_9Y_2. However, using these same two peptides with the isolated recombinant α subunit, the potency of both peptides is the same (Figure 6B).

DISCUSSION

The bacterial expression and purification of the recombinant α and β subunits of the *X. laevis* casein kinase II are described. Both subunits obtained by this procedure are functional and can reconstitute a fully active holoenzyme when they are incubated in a standard assay mixture in stoichiometric amounts. The β subunit can stimulate about 5-fold the activity of α, which has a basal specific activity which is similar to that reported for recombinant α from human and drosophila (Grankowski et al., 1991; Lin et al., 1991).

The availability of these purified recombinant subunits of CKII opens up the possibility of studying the interaction of these two polypeptides and of exploring the structural features that are important for their biological activity.

It has long been known that the β subunit of CKII was autophosphorylated (Hathaway & Traugh, 1979; Meggio & Pinna, 1984). However, the lack of knowledge of the amino acid sequence of β and the low amounts of the subunit available made it difficult to define the precise autophosphorylation sites and to determine whether this reaction was important for enzyme activity. More recently, the laboratory of Krebs reported experiments that indicated that the principal autophosphorylation sites were serines in positions 2 and 3 of the β subunit and that this site was also phosphorylated *in vivo* (Litchfield et al., 1991). However, no evidence was presented as to the possible functional effect of this phosphorylation. Using recombinant β subunit, Boldyreff et al (1992) have made deletion mutants that eliminate different parts of the molecule, including an amino-terminal peptide containing the autophosphorylation site. This deleted protein was found to be as active as the wild-type peptide in activating α. Using a different approach, we have specifically mutated serines-2

and -3 to glycines in the recombinant β subunit. This mutant completely lacked the capacity to be phosphorylated by α but was active in stimulating the catalytic activity of α. These results confirm the identification of the autophosphorylation site and establish that autophosphorylation of β is not necessary for the stimulatory capacity of this protein.

There have been reports that indicate that phosphorylation of the β subunit can cause activation of CKII. Ackerman et al. (1990) reported that EGF stimulation of CKII was correlated to the phosphorylation state of the β subunit. Alkaline phosphatase treatment decreased the CKII level of activity to the basal levels found in untreated cells. However, this work did not identify the sites of phosphorylation in the β subunit nor the kinases responsible for their phosphorylation. Mulner-Lorillon et al. (1990) published the observation that $p34^{cdc-2}$ kinase from starfish eggs could phosphorylate the β subunit of CKII from *X. laevis in vitro* and that this phosphorylation caused an activation of CKII. Lichtfield et al. (1992) have confirmed the phosphorylation of CKII β by $p34^{cdc-2}$ and have shown that, both *in vivo* and *in vitro*, the site phosphorylated is serine-209 in the human CKII enzyme. This work, however, did not define any effect of this phosphorylation on enzyme activity.

The availability of the recombinant β subunit will now provide an opportunity for systematic testing of the possible regulatory effects of phosphorylation by other kinases of the other serine, threonine, and tyrosine residues present in this protein.

In this report, data are presented for two methods for the study of the interaction of the CKII α and β subunits. One approach uses a byproduct of the expression of the β subunit: the fusion protein that contains at its amino end the sequence of glutathione transferase and at its carboxy end the β subunit of CKII. We observe that this fusion protein has the capacity to interact with α and to activate it. Obviously, this fusion protein can use its glutathione transferase moiety to bind to glutathione–Sepharose beads. These beads, therefore, make it possible to immobilize the chimeric protein and, subsequently, to bind the α subunit to this solid matrix. This method could permit the study of the effects of mutations in α and β subunits on the interaction of the two subunits, independent of activity measurements.

Similarly, the stable adsorption of the α subunit to glass fiber filters can be useful since the bound subunit is active. Although no information was obtained on the nature of the interaction of this protein with the glass fibers, it was observed that the activity retained on the filters was a function of the concentration of the enzyme used in the incubation with the filters. It was also oberved that the binding occurred at 4 °C and high concentration of salt (0.7 M NaCl). The activity of immobilized α retained its characteristic property of being inhibited by heparin. The most interesting observation made with the α subunit bound to glass fiber filters, however, was the fact that it could be activated by β to a degree similar to that observed in solution.

Conclusive evidence has been presented by several workers that the isolated α subunit of CKII is a monomer at salt concentrations ranging from 0.6 to 2 M (Hu & Rubin, 1990b; Lin et al., 1991). When β is added to α, however, the $\alpha_2\beta_2$ tetramer is formed. Presumably, therefore, α is immobilized on the glass fiber matrix as a monomer, and it that state, the formation of the tetramer would be impeded. The fact, therefore, that β can activate immobilized α would suggest that tetramer formation is not required for the stimulus of the catalytic activity by β and that an $\alpha\beta$ heterodimer might be fully active.

The availability of isolated recombinant α subunits permits one to study the effect of the β subunit on the recognition of protein and peptide substrates and inhibitors. Several reports have recently shown that the relative affinity and specificity of the α subunit for protein and peptide substrates are different from that of the tetrameric holoenzyme (Meggio et al., 1992; Grankowski et al., 1991). A striking difference was described in the case of calmodulin which can only be phosphorylated by the holoenzyme in the presence of polylysine; however, calmodulin is readily phosphorylated by isolated α in the absence of this polycation.

In this report, the recognition of peptides containing tyrosine and glutamic acid has been studied with the recombinant subunits. As previously shown using the naturally occurring holoenzyme, the presence of tyrosine residues in glutamic acid rich copolymers enhances the inhibitory capacity of the acidic peptide by 2 orders of magnitude. As presented above, the enhancement of the inhibition caused by tyrosine is reduced by 1 order of magnitude when the isolated α subunit is used.

It is also interesting that the position of the tyrosine residues within the poly(glutamic acid) cluster is recognized by the holoenzyme. Thus, using synthetic peptides and the holoenzyme, it was demonstrated that an undecapeptide that has two tyrosine residues in the amino-terminal end is a stronger inhibitor than a peptide of the same composition but with the aromatic amino acids at its carboxyl end. Similar positional effects were noted for acidic hexapeptides containing tyrosine in relation to their efficiency in inhibiting the CKII holoenzyme from rat liver (Marin et al., 1986); again, the N-terminal position of tyrosine was most effective. The isolated α subunit, on the other hand, fails to discriminate between these two structural conformations.

In addition to the interest of this finding of a role for the β subunit in the recognition of substrate and inhibitory sequences, the result obtained with the holoenzyme is important in itself. The favored position of the tyrosine residues in the amino-terminal side of the polyglutamic cluster is similar to the preferred position of the seryl and threonyl residues in substrate sequences (Meggio et al., 1984). It seems possible, therefore, to postulate that Tyr-X-X-X sequences (where X is an acidic amino acid or a phosphorylated amino acid) may act as pseudosubstrate inhibitors. This would imply that CKII is recognizing tyrosyl residues as related to serine and threonine. The recent finding of dual-specificity protein kinases capable of phosphorylating tyrosine residues as well as serine/threonine (Kosako et al., 1992) may be pertinent to this observation. However, CKII and the dual-specificity kinases do not share sequence similarities other than those sequences common to all protein kinases. The fact that CKII, like tyrosine kinases, can efficiently use Mn^{2+} or Co^{2+} as well as Mg^{2+} (Gatica et al., 1993) also supports the speculation that CKII might be a protein kinase on the evolutionary path that leads to these ambivalent or dual-specificity protein kinases.

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