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Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes

JOIE ROWLES*, CLIVE SLAUGHTER†, CAROLYN MOOMAW†, JOAN HSU†, AND MELANIE H. COBB*

*University of Texas Southwestern Medical Center, Department of Pharmacology, 5323 Harry Hines Boulevard, Dallas, TX 75235-9041; and †Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235

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ABSTRACT **We have purified casein kinase I (CKI) over 6000-fold from bovine thymus and have sequenced seven tryptic peptides that account for nearly 25% of the primary sequence of the enzyme. By using PCR, partial cDNAs encoding CKI and a related enzyme (CKI-δ) were isolated. A product that may correspond to an alternatively spliced form of CKI was also detected. The CKI PCR product was used to probe a bovine brain cDNA library from which cDNAs corresponding to CKI (CKI-α) and two homologous enzymes (CKI-β and CKI-γ) were identified. The finding that there are at least four CKI-like enzymes suggests that CKI activity in tissues or cell extracts may be composed of multiple related but distinct protein kinases. This group of enzymes is not similar to any other known protein kinases and may, therefore, represent an additional branch of the protein kinase family.**

Casein kinase I (CKI) is a ubiquitous serine/threonine-specific protein kinase that was first described over 15 years ago (1–4). CKI, casein kinase II (4), and glycogen synthase kinase 3 (5) comprise the majority of protein-serine/threonine kinase activities in cell extracts that recognize acidic rather than basic residues in their substrates. Although some candidate physiologic substrates of CKI have been identified (6–8), biochemical analysis has offered few clues about its functions or its regulation. Nevertheless, multiple types of regulation have been proposed. First, its activity may be directly stimulated by hormones (9–11) or viral transformation (12). Extracts of insulin-treated and of virally transformed cells have been reported to contain elevated CKI activity (9, 12). Second, its activity may be regulated by phosphorylation of its substrates (13); certain proteins, such as glycogen synthase (14), become substrates for CKI only after they have been phosphorylated by other kinases. Thus, a phosphorylated residue can create the determinant required for recognition of substrates by CKI. Third, its activity is inhibited by phosphatidylinositol 4,5-bisphosphate (15, 16). This suggests a mechanism for inhibitory control of the enzyme by agents that regulate phosphatidylinositol turnover. CKI also interacts with the cytoskeleton as well as membranes and nuclei (1, 6, 7, 17); thus, its association with substrate-bearing organelles may provide another means for its regulation.

To facilitate studies of CKI, we have purified the enzyme to high specific activity and in sufficient quantities to sequence. From the tryptic peptide sequence, oligonucleotides were synthesized for use as primers in PCR by using bovine brain cDNA as a template. One of the resulting PCR products was used to isolate cDNAs encoding CKI and related enzymes.‡

MATERIALS AND METHODS

Casein Kinase Assay. Casein kinase activity was assayed in a final volume of 50 μl containing bovine serum albumin at 20 mg/ml, α_s-casein (United States Biochemical) at 0.5 mg/ml, 0.1 M NaCl, 15 mM Hepes (pH 8.0), 100 μM [γ-^{32}P]ATP (500–3000 cpm/pmol), and 5 mM $MgCl_2$. After 7 min at 30°C, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid in 5 mM sodium pyrophosphate. Precipitates were collected and assayed for radioactivity as described (18). Activity in the absence of exogenous casein was subtracted from activity measured in its presence. To assess activity contributed by casein kinase II in lysates, duplicate 15-min assays were performed by using 10 μM ATP with or without 100 μM unlabeled GTP. Activity lost in the presence of GTP was attributed to casein kinase II. Other methods were as in ref. 18.

Purification of CKI. Bovine thymus (≈300 g) was homogenized in 2.4 volumes of buffer [50 mM Tris·HCl (pH 7.5), 50 mM EDTA, 1 mM dithiothreitol, 100 mM ammonium sulfate, 10 mM benzamidine, and 15% (vol/vol) glycerol] in a Waring blendor for 2 min and then with a Polytron for 1 min. The homogenate was centrifuged in a Beckman JA-14 rotor at 12,200 rpm for 45 min. Proteins insoluble in ammonium sulfate (45% saturation) were collected by centrifugation in a Beckman type 35 rotor at 31,000 rpm for 60 min, resuspended in buffer B [50 mM Tris·HCl (pH 7.5), 15 mM 2-mercaptoethanol, 2.5 mM benzamidine, 0.1 mM EDTA, and 10% glycerol], diluted until the conductivity was 12–15 mS, and mixed with ≈350 ml of DEAE-cellulose in buffer B containing 50 mM ammonium sulfate. After 30 min, the resin was filtered and washed with 200 ml of buffer B. The protein not adsorbed and the wash were combined with 400 ml of phosphocellulose for 45 min. The resin was collected by filtration, washed with 500 ml of buffer B, resuspended in 500 ml of buffer B containing 0.35 M NaCl, poured into a column (5 × 15 cm), and developed with another 500 ml of 0.35 M NaCl in buffer B. The CKI activity was then eluted with a 2-liter gradient of 0.35–1.2 M NaCl in buffer B. The activity eluting at about 0.4 M NaCl was pooled and dialyzed against 4 liters of buffer B overnight. The dialysate was applied to an S-Sepharose column (2.3 × 36 cm), washed with 500 ml of 0.2 M NaCl in buffer B, and developed with a 1-liter gradient of 0.2–1.2 M NaCl in buffer B. The activity that eluted at 0.32 M NaCl was pooled, diluted with buffer B to a NaCl concentration <0.15 M, and applied to phosvitin-Sepharose. The column (2.5 × 10 cm) was washed with 150 ml of 0.32 M NaCl in buffer B and developed with a 250-ml gradient of 0.32–1.2 M NaCl in buffer B. The activity eluting at ≈0.4 M NaCl was applied to Sepharose S-200 (2.5 × 94 cm column) in 0.2 M NaCl in buffer B. The single peak of CKI activity was applied directly to hydroxylapatite (0.7 × 0.5 cm column) in 0.2 M NaCl in buffer B. CKI passed through while the impurities were retained on the resin. The enzyme was concentrated on phosphocellulose (0.5 × 0.7 cm column) and eluted with 50

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Abbreviation: CKI, casein kinase I.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M76542–M76545 for CKI-γ, -α, -β, and -δ, respectively).

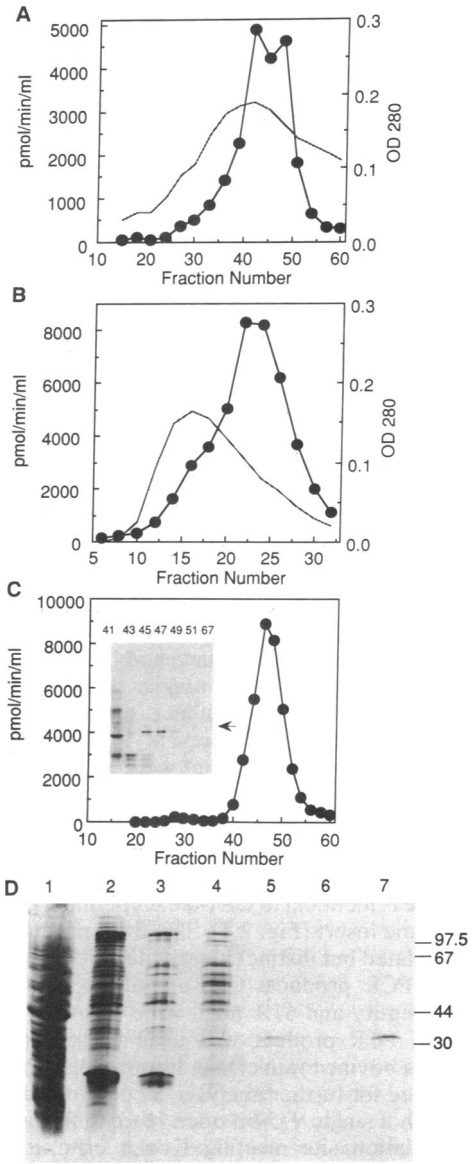

FIG. 1. Chromatography of CKI on S-Sepharose (*A*), phosvitin-Sepharose (*B*), and Sephacryl S-200 (*C*). Casein kinase activity (●) is shown. The absorbance at 280 nm is indicated for the S-Sepharose and phosvitin-Sepharose columns. (*C Inset*) Silver-stained gel of the fractions containing the CKI activity. The arrow indicates the band corresponding to CKI. (*D*) Silver-stained gel of CKI at various stages of purification. Aliquots of pools from each step were analyzed by electrophoresis on a 10% polyacrylamide gel and silver-stained. Lane 1, DEAE-cellulose; lane 2, phosphocellulose; lane 3, S-Sepharose; lane 4, phosvitin-Sepharose; lane 5, Sephacryl S-200; lane 6, hydroxylapatite; lane 7, phosphocellulose.

mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, 0.8 M NaCl, 0.5 mM $MgCl_2$, and 45% glycerol.

Protein Sequence Analysis. Purified enzyme (≈45 μg) was precipitated with 2 volumes of ethanol, resuspended in 1% ammonium bicarbonate, and digested with trypsin. Peptides were chromatographed on a 2.1 × 100 mm Applied Biosystems RP-300 column in 0.1% trifluoroacetic acid (19) and rechromatographed on the same column at pH 6.5. Peptides were sequenced using an Applied Biosystems model 470A sequencer according to manufacturer's specifications. Of the seven peptides sequenced, only one (DIKPDNFLMGIGR) was obviously derived from the kinase domain (20).

Isolation and Sequencing of cDNAs. Degenerate oligonucleotides were synthesized based on peptide sequence and used as primers to amplify bovine brain cDNA by PCR. PCR products obtained using oligonucleotides based on DNFLMG(5′) and YAFINA(3′) as primers were subcloned into pGEM (Promega) and sequenced with Sequenase version 2 according to suggested procedures (United States Biochemical). A size-selected [2–4 kilobases (kb)] bovine brain cDNA library constructed in λZap (Stratagene) was screened at 52°C in 2× standard saline citrate with one PCR product of 159 base pairs (bp). The inserts from the phage clones were recovered as Bluescript plasmids, and both strands were sequenced. Other methods were as in Sambrook *et al.* (21).

RESULTS

The CKI purification scheme improves upon two previously published methods (22, 23). Adsorption to DEAE-cellulose removes the majority of casein kinase II, accounting in part for the loss of units at this first step. The material that flows through DEAE-cellulose elutes from phosphocellulose as one peak of casein kinase activity at 0.4 M NaCl. Chromatography on S-Sepharose (Fig. 1*A*) removes small protein contaminants that are not completely resolved by gel filtration or on hydroxylapatite, resulting in a 5-fold purification. After chromatography on phosvitin-Sepharose (Fig. 1*B*) and Sepharose S-200, CKI is 10–30% pure (Fig. 1*C Inset*). An ≈6-fold further purification is achieved by applying the enzyme in 0.2 M NaCl to hydroxylapatite; contaminants are retained, while the enzyme flows through. A small amount of a 66-kDa protein sometimes found in the eluate is removed by the final phosphocellulose column, accounting for the slight increase in purity at this step. The resulting CKI is homogenous as judged by the appearance of one silver-stained band (Fig. 1*D*) of 35 kDa. The yield is from 50 to 100 μg per 100 g of thymus. The specific activity of calf thymus CKI (Table 1), 1–2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, is slightly higher than reported previously (22, 23).

Degenerate oligonucleotides, one set of which represented sequence from protein kinase subdomain VI as defined by Hanks *et al.* (20) (see *Materials and Methods*), were used as primers to amplify bovine brain cDNA by PCR. Three PCR

Table 1. Purification of casein kinase from bovine thymus

Sample	Protein, mg	Activity, units*	Specific activity, units/mg	Recovery, %	Purification, fold
23,300 × *g* supernatant	10,000	2200	0.22	100	1
45% $(NH_4)_2SO_4$	10,300	2700	0.26	123	1.2
DEAE flow-through	10,100	1300	0.13	61	0.6
Phosphocellulose	330	1450	4.4	66	20
S-Sepharose	36	810	22	37	100
Phosvitin-Sepharose	8	410	48	19	220
Sephacryl S-200	2	290	140	13	650
Hydroxylapatite flow-through	0.3	270	900	12	4100
Phosphocellulose	0.2	310	1400	14	6400

*One unit of activity is defined as the amount of enzyme that transfers 1 nmol of phosphate to α_s-casein per min under the specified assay conditions.

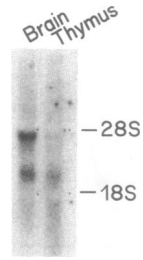

FIG. 3. Northern analysis. RNA was purified from bovine brain and thymus by using guanidine thiocyanate (21). Total RNA (10 μg) was separated on a 1% agarose/6.3% formaldehyde gel, transferred to a nylon membrane (GeneScreen*Plus*; NEN), and probed with a 725-bp *Eco*RI/*Pst* I fragment of CKI-α labeled with [α-^{32}P]dCTP by random priming. The membrane was washed in 0.1× standard saline citrate for 45 min at 65°C and exposed to XAR-5 film with intensifying screens at −80°C for 2 days. The sizes of the hybridizing bands were determined by using 28S (4.4 kb) and 18S (1.97 kb) ribosomal RNA as markers. Ethidium bromide staining of the gel before transfer indicated that the ribosomal RNA bands from brain and thymus were of equal intensity.

products, two of 159 bp and one of 243 bp, were sequenced and found to span subdomains VI–VIII of the protein kinase (Fig. 2*A*). One of the 159-bp products and the 243-bp product (designated CKI-α and CKI-αL) appear to be derived from CKI because they contain sequence encoding the three additional amino acids (IGR) present in the CKI tryptic peptide (underlined in Fig. 2*B*) that were not used to create the 5′ primer. These products were likely derived from protein kinases because they contained the DFG characteristic of subdomain VII (20). The longer product (CKI-αL) may represent an alternately spliced form of CKI; its nucleotide sequence is identical to CKI-α except that it contains an 84-base in-frame insert (Fig. 2*A*). The third product appears to encode a related but distinct kinase (designated CKI-δ; see below). The PCR products CKI-α and CKI-δ share 79% amino acid identity and 67% nucleotide identity.

The CKI-α PCR product was used to screen 5×10^5 plaques from a bovine brain cDNA library. Of 14 hybridizing plaques purified for further analysis, 1 contained an insert of ≈1400 bp with a single 975-bp open reading frame (Fig. 2*B*) and a 5′ initiation site meeting Kozak criteria (24). The deduced amino acid sequence (325 residues) predicted a protein of 37.6 kDa, which is close to the size estimated for CKI by SDS/PAGE. It contained the 11 conserved subdomains of protein kinases described by Hanks *et al.* (20) and all seven of the CKI tryptic peptides (underlined in Fig. 2*B*). Thus, this cDNA is likely to be CKI and we designated it CKI-α. Another cDNA (CKI-αL) was isolated that is identical to CKI-α over the ≈600 bp sequenced, except for an in-frame insertion that matches that in the longest of the three PCR products noted above. This cDNA likely corresponds to either an alternatively spliced form or a partially processed form of CKI-α.

To examine the size of the CKI-α mRNA, a 725-bp fragment (corresponding to amino acids 10–253) of the CKI-α cDNA was generated, which hybridized to two major tran-

FIG. 2. Sequences of CKI-like enzymes. (*A*) Sequences of three PCR products, CKI-α, CKI-αL, and CKI-δ. The sequence of PCR products spanning subdomains VI–VIII is shown. Identical residues are indicated by dots. (*B*) Nucleotide and predicted amino acid sequence of CKI-α. Tryptic peptides are underlined. One tryptic peptide in subdomain VIII contained an ambiguous residue originally thought to be phenylalanine, which is serine in the cDNA. (*C*) Alignment of CKI-α with CKI-β and CKI-γ. Dots represent identical amino acids. Gaps, represented by hyphens, have been inserted to improve the alignment. Subdomains conserved in protein kinases (20) are indicated by Roman numerals.

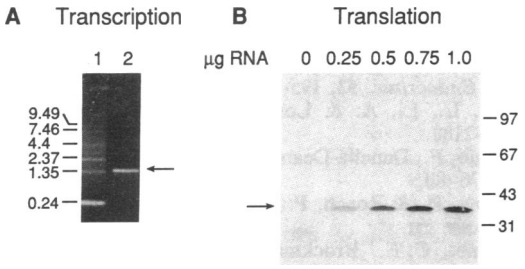

FIG. 4. Transcription and translation of CKI-α *in vitro*. The Bluescript vector containing the CKI-α insert was linearized with *Bam*HI (cutting at the 3′ end of the insert) and treated with proteinase K (50 μg/ml). (*A*) Approximately 500 ng was transcribed using a Riboprobe kit (Promega) according to manufacturer's specifications. After 1 hr at 39°C, the reaction was extracted with phenol/chloroform, 1:1, and precipitated with ethanol. An aliquot of the resulting RNA is shown in lane 2 along with a 0.24- to 9.5-kb RNA ladder (BRL) as markers (lane 1). (*B*) Increasing amounts of RNA were translated using a nuclease-treated Promega rabbit reticulocyte lysate in the presence of [^{35}S]methionine. After 1 hr at 30°C, aliquots were analyzed by SDS/PAGE and exposed to XAR-5 film for 2 days at −80°C. The autoradiogram is shown. Molecular size markers (in kDa) are indicated at right.

scripts of 4.1 and 2.2 kb in bovine brain and one major transcript of 2.2 kb in bovine thymus (Fig. 3). The 4.1-kb RNA was also faintly visible in thymus.

To provide additional data that the CKI-α cDNA encodes a casein kinase, the cDNA was transcribed *in vitro*, and the resulting mRNA was translated in a rabbit reticulocyte lysate. An mRNA of 1.4 kb was generated (Fig. 4*A*), which directed the synthesis of a protein the size of CKI, ≈35 kDa (Fig. 4*B*). Because reticulocyte lysates contain high CKI activity, we were unable to measure an increase in activity in the RNA-primed lysates relative to the control lysates. Therefore, to show that this clone encodes an active enzyme, a recombinant baculovirus was made (25) using a pVL1393 transfer vector (Invitrogen) into which the CKI-α cDNA had been ligated. Cells infected with virus from three recombinant plaques displayed CKI activity that ranged from 2.8- to 7.8-fold higher than the CKI activity in uninfected cells (Fig. 5).

Amino acid sequences predicted by other clones isolated from the cDNA library using the CKI-α probe encode two protein kinases [designated CKI-β and CKI-γ (Fig. 2*C*)] related to but distinct from CKI-α and CKI-δ. The CKI-β sequence is a composite of sequence from two overlapping cDNA clones. One of the CKI-β clones contained an open

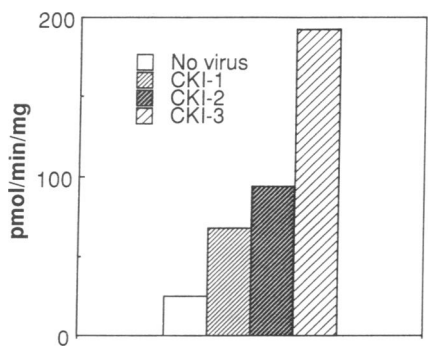

FIG. 5. Expression of CKI-α in Sf9 cells. The CKI-α cDNA with 62 bp of 5′ untranslated sequence was cloned into the baculovirus transfer vector pVL1393 and cotransfected onto Sf9 cells with baculoviral DNA from strain AcRP23-LacZ that had been linearized with *Bsu*36I as described (25). Three recombinant viruses were selected by plaque assay and used to infect 1.5 × 10^6 Sf9 cells. After 4.5 days, cells were homogenized and CKI activity in 100,000 × *g* supernatants was assayed. Protein concentrations of all four supernatants were within 10%.

reading frame encoding subdomains III–XI of the kinase and included a stop codon; the other CKI-β clone contained a 5′ initiation site and an open reading frame encoding subdomains I–VIII of the kinase and was identical to the first clone throughout the 327 overlapping nucleotides that were sequenced. The deduced sequence of CKI-β contains 336 amino acids predicting a protein of 38.7 kDa, is 78% identical to CKI-α, and has the same number of residues prior to the ATP binding site as CKI-α with an 11-amino acid extension at the C terminus.

An additional clone with an insert of 1175 bp is designated CKI-γ and also contains a likely 5′ initiation site that fits the Kozak consensus (24); however, this clone is incomplete and ends in kinase subdomain X. Over the shared lengths of available sequence (260 amino acids from the N terminus to subdomain X), CKI-γ is 52% identical to CKI-β and 56% identical to CKI-α. Neither of these cDNAs (β or γ) is identical to the third PCR product, CKI-δ. From the small amount of sequence predicted by the CKI-δ PCR product, it appears to be more closely related to CKI-α than to CKI-β or CKI-γ.

DISCUSSION

We have isolated a cDNA that encodes CKI. The evidence supporting this conclusion is as follows: the protein predicted from the cDNA contains all of the tryptic peptides identified by protein sequencing, an mRNA was transcribed from the cDNA *in vitro* that directed the synthesis of a 35-kDa protein, and the protein expressed in Sf9 cells from recombinant baculovirus containing the cDNA had casein kinase activity.

Complete or partial cDNAs have been isolated that likely represent four distinct genes encoding CKI-like enzymes, designated CKI-α, CKI-β, CKI-γ, and CKI-δ. These four enzymes are much more closely related to each other (>50% identity) over their entire lengths than to any other known protein kinases (≤21% identity), even if comparisons are restricted only to their protein kinase domains (20). This includes two other serine/threonine kinases preferring acidic substrates, casein kinase II (26) and glycogen synthase kinase 3 (27), suggesting that substrate specificity is not reflected in the overall similarity of the primary sequence. Interestingly CKI-α and its homologs contain a longer insert between subdomains VII and VIII than is present in any other kinase except CDC7, a kinase required for replication in yeast (28). The lengths of other insert regions are also unusual and conserved among the CKI enzymes (e.g., a long insert between subdomains X and XI and a short span from subdomain VIII to subdomain IX). Because none of these CKI enzymes is more than ≈20% identical to any other protein kinase, little can be inferred about CKI from its relationships to other proteins. We conclude that the CKI enzymes comprise an additional branch of the protein kinase family.

The CKI-α gene may give rise to two proteins due to alternative splicing. One would contain a novel insertion of 28 amino acids in-frame between subdomains VI and VII.

Few reports in the literature have presaged the existence of multiple CKI-like enzymes. CKI activity has been nearly universally described as a monomeric enzyme of ≈34–38 kDa with very similar chromatographic properties whatever its source. However, some investigators have reported dimeric forms (29), a broad range of sizes for the monomeric form (30), and association of the enzyme with subcellular organelles (1, 6, 7, 15–17) as well as discrepancies in substrate specificity and effects of cations on activity (30). Isolation of cDNAs encoding CKI enzymes should enable us to generate antibodies to distinguish among the forms and localize them to their respective intracellular sites, to generate oligonucleotide probes to study their expression in tissues and during development, and to express the individual forms to study

their biochemical properties. All of these approaches should be useful in defining the biochemical similarities and differences and the functions and regulation of these protein kinases.

Note Added in Proof. Recently, a yeast protein kinase that may be involved in DNA repair, HRR25, has been identified (31) that is 60% identical to CKI-α over its catalytic domain.

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