Casein Kinase I α and αL: Alternative Splicing-Generated Kinases Exhibit Different Catalytic Properties[†,‡]

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ABSTRACT: Casein kinase I (CKI) is a family of serine/threonine protein kinases found in all eukaryotes examined to date. Here, the rat CKI isoforms α and αL were cloned and expressed in both eukaryotic and prokaryotic systems. Characterization of the genomic DNA flanking the exon unique to CKIαL demonstrated that CKIα and CKIαL arise by the alternative splicing of a common pre-mRNA molecule. To the best of our knowledge, the αL isoform is the only known active serine/threonine kinase to contain an insert within its catalytic domain. Tissue distribution of each splicing isoform was examined by RT−PCR, immunoprecipitation, and Western blotting. Both isoforms were expressed in all tissues tested but at different levels. Bacterially expressed CKIα isoforms were active and therefore biochemically characterized. CKIα and CKIαL proteins were demonstrated to have casein kinase I catalytic properties. More importantly, the recombinant isoform proteins exhibited differences in binding and activity toward common CKI substrates. These observations demonstrate that the αL insert within the kinase domain modulates substrate kinetics. These kinetic differences suggest that CKIα and CKIαL may perform different biological roles.

Casein kinase I (CKI)[1] represents a family of second messenger-independent serine/threonine protein kinases originally defined by their ability to use ATP, but not GTP, and to prefer acidic proteins like casein and phosvitin as substrates (Tuazon & Traugh, 1991; Dahmus, 1981a). As far as more pertinent targets are concerned, many substrates for CKI have been determined *in vitro* such as SV40 large T antigen (Graser *et al.*, 1988; Cegielska & Virshup, 1993), RNA polymerases I and II (Dahmus, 1981b), p53 (Milne *et al.*, 1992), glycogen synthase (Ashmad *et al.*, 1984; Flotow & Roach, 1989, 1991), the insulin receptor (Tuazon *et al.*, 1985), and spectrin (Simkowski & Tao, 1980). Of these, only SV40 large T antigen and glycogen synthase are known to be phosphorylated by CKI *in vivo*. As an activity, the kinase has been detected in the cytoplasm and the nucleus and associated with membranes. The cytosolic and membrane-associated forms of CKI appear to be monomers of 30−37 kDa (Ashmad *et al.*, 1984; Kitagawa & Racker, 1982). Nuclear forms of the enzyme have apparent molecular masses ranging from 25 to 55 kDa (Thornburg *et al.*, 1978; Rikans & Ruddon, 1976). CKI exists in all species and cell types examined (Tuazon & Traugh, 1991). Indirect immunofluorescence studies using CKI-specific antibodies have demonstrated that CKI is associated with the centrosome and cytosolic vesicular structures in interphase cells and with the mitotic spindle (Brockman *et al.*, 1992). More recently, the association of CKI with synaptic vesicles (Gross *et al.*, 1995) and neurofilaments (Link *et al.*, 1993) has been reported.

Since 1991, the cloning of CKI isoforms from yeast and mammals has permitted the use of molecular biology to more clearly define the function and regulation of this enzyme. In *Saccharomyces cerevisiae*, three isoforms of CKI -- HRR25, YCK1, and YCK2—have been cloned, sequenced, mutated, and shown to be essential for growth and viability (Hoekstra *et al.*, 1991; Robinson *et al.*, 1992; DeMaggio *et al.*, 1992). HRR25 is involved in the regulation of DNA repair (Hoekstra *et al.*, 1991), whereas YCK1 and YCK2 are required for bud morphogenesis and cytokinesis (Robinson *et al.*, 1993). In mammals, full-length cDNAs for CKIα and CKIβ have been isolated from bovine brain (Rowles *et al.*, 1991), as well as CKIγ, -δ, and -ϵ isoforms from rat testis (Graves *et al.*, 1993; Fish *et al.*, 1995; Lanmin *et al.*, 1995). CKIδ and -ϵ appear to be most like HRR25 in terms of both sequence and function, and both can complement its function in deletion mutant strains (Fish *et al.*, 1995). In addition, CKIγ, which exists as three genetically distinct isoforms, can restore viability to a Δyck1/yck2^{ts} double-mutant strain under the nonpermissive condition (Lanmin *et al.*, 1995). The exact roles of CKIα and -β remain undefined. The reported range of molecular masses and subcellular localizations may be explained in part by the large number of CKI isoforms.

The activity of CKI can be regulated in several ways. Phosphatidylinositol 4,5-bisphosphate (PIP_2) has been shown to inhibit CKI's activity (Brockman & Anderson, 1991; Bazenet *et al.*, 1990). Activation of CKI is observed with some hormones, such as insulin in certain cell types (Cobb & Rosen, 1983). Furthermore, the phosphorylation and dephosphorylation of substrates provide a powerful means for directing the activity of CKI toward particular targets. For example, dephosphorylated glycogen synthase is a poor

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[‡] The rat casein kinase I α and αL sequences have been deposited in Genbank as U77582 and U77583, respectively.

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[1] Abbreviations: CKI, casein kinase I; PCR, polymerase chain reaction; RT−PCR, reverse transcription−polymerase chain reaction; bp, base pairs; kbp, kilobase pairs; IPTG, isopropyl 1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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substrate for the enzyme. However, CKI can phosphorylate a glycogen synthase molecule that has been previously phosphorylated by other kinases (Flowtow & Roach, 1989). Finally, as discussed here, at least for certain isoforms in this kinase family, their activity is regulated at the level of alternative splicing.

From the molecular genetic work described above, it has been determined that the α isoform may exist as at least three, and potentially four, splicing variants. A partial CKIαL cDNA has been reported containing an in-frame insertion of 84 nucleotides encoding 28 amino acids inserted just after Lys_{152} (Rowles *et al.*, 1991). In addition, a human isoform of CKIα, termed CKIα2, containing an extra 36 nucleotides in-frame very near its 3′ end has also been described (Fish *et al.*, 1995). Preliminary evidence also suggests that some kinase molecules may contain both inserts. These splicing isoforms could have altogether different biochemical properties and subcellular localizations. Studying specific isoforms could therefore prove important in defining the exact regulatory role(s) of the CKIα subfamily of enzymes in higher eukaryotes.

The present studies were initiated to understand how the CKIα and CKIαL isoforms were generated. In addition, we were interested in the biochemical consequences of the addition of this 28-amino acid insert which lies within the catalytic domain. We subsequently cloned and sequenced the entire coding sequences of rat brain CKIα and CKIαL as well as a stretch of genomic DNA containing the αL exon. Analysis of the genomic DNA sequences flanking the CKIαL exon indicates that these two isoforms are produced by alternative splicing. To varying degrees, the transcripts and gene products of these isoforms exist in all tissues. Finally, isoform proteins exhibit kinase activities with different substrate kinetics. The repercussions of these differences is then discussed.

MATERIALS AND METHODS

General. Casein, phosvitin, glycogen synthase, heparin, glutathione−agarose, and protein A−Sepharose were obtained from Sigma. CKI-7 was purchased from Seikagaku America. The peptide used for antibody production was synthesized and purified (≥95% purity) by Multiple Peptide Systems. Oligonucleotides for PCR were synthesized by the University of Wisconsin Biotechnology Center. AmpliTaq and KlenTaq DNA polymerases were obtained from Perkin Elmer-Cetus and Ab-Peptides, respectively. Restriction enzymes, T4 DNA ligase, AMV reverse transcriptase, DNA polymerase I Klenow fragment, alkaline phosphatase-conjugated secondary antibody, and nucleotides were products of Promega. Sequenase was purchased from USB. Vectors pBluescript SK+ and pcDNA3 were acquired from Stratagene and Invitrogen, respectively. Vectors pMAL and M13mp18/19 were supplied by New England Biolabs. Adult rat brain cDNA was obtained from Clontech. [^{35}S]-dATP was provided by Amersham and [γ-^{32}P]ATP by DuPont-New England Nuclear, and [^{125}I]protein A a product of ICN or Amersham. GST−SV40 large T antigen constructs were gifts from Dr. A. G. Wildeman and were purified in our laboratory. RPMI media, fetal bovine serum, and horse serum were obtained from GIBCO-BRL.

Production of Polyclonal Antibodies Against the CKIαL Isoform. The peptide, ESPVGKRKRSMTVSPSQDC, corresponding to amino acids 3−20 of the rat brain CKIαL unique sequence with an additional cysteine residue at the C terminus, was coupled to keyhole limpet hemocyanin (KLH) (Tamura *et al.*, 1983) using an (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide ester (Pierce). Rabbits were immunized twice with the KLH−peptide (200 μg each time) and boosted intravenously with 50 μg of KLH−peptide. A column was prepared by coupling the peptide to Affi-Gel 10 (Bio-Rad) as per the manufacturer's instructions. Crude sera were affinity-purified using this column. A polyclonal antibody was also raised against a rat CKIαL maltose binding protein fusion. The CKIαL fusion protein was prepared by inserting the entire coding sequence of the rat CKIαL cDNA into pMAL-C2 in-frame relative to the maltose binding protein coding sequence and expressing the resultant construct in *Escherichia coli* DH5α (Ny & Bjork, 1980). This CKIαL fusion protein was purified on an amylose affinity column (NEB) and a hydroxyapatite column following the instructions of the manufacturer. A rabbit was immunized twice with the fusion protein (0.3 mg each time) followed by a boost with 0.1 mg. The crude sera were affinity-purified on the peptide column as described previously (Brockman *et al.*, 1992). The specificities of both antibodies were tested by immunoprecipitation and Western blotting of *E. coli* expressing CKIα or CKIαL fusion proteins or of PC12 rat pheochromocytoma cell lines (Greene & Tischler, 1976).

PCR and DNA Sequencing of Rat Brain Complementary and Genomic DNA. Adult rat brain cDNA was amplified by PCR to isolate CKIα isoforms from rat brain. The 5′ amplimer 5′-TCTTCGTCTC**CT**AGGATGGCGAGCAG-CAGCG-3′ corresponds to nucleotides 379−411 of bovine brain CKIα cDNA (GenBank accession no. M76543), except for the nucleotides in bold typeface, which were changed in order to generate an *Avr*II restriction site. The 3′ amplimer 5′-GATTCATGCTTAGAAACCTGTGG-3′ corresponds to nucleotides 1358−1380 of the bovine brain CKIα cDNA. Underlined portions of sequences in both amplimers represent the nucleotides within the coding regions, while the rest of the sequences represent the 5′- or 3′- untranslated regions. The PCR products were purified by agarose gel electrophoresis and subcloned into M13mp18/19 or pBluescript, and both strands were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Rat genomic DNA was isolated from the brain of a male rat and amplified with two pairs of primers. The first pair consisted of the forward primer 5′-AATGGGTATTGGGCGTCACTG-3′ (nucleotides 429−449 of rat CKIαL nucleotide sequence) and the reverse primer 5′-CTGGTTTAATCCTGAGAAAGATGG-3′ (nucleotides 517−540). The second pair was comprised of the forward primer 5′-TGTTTAGAATCTCCAGTGGG-3′ (nucleotides 457−476 of the rat CKIαL sequence) and the reverse primer 5′-TGTTGCCTTGTCCTGTTGTCTC-3′ (nucleotides 578-599). The PCR products were purified and sequenced as before (Sanger *et al.*, 1977).

Expression of Rat Brain CKIα and CKIαL cDNAs in E. coli. Rat brain CKIα and CKIαL cDNAs obtained by PCR were filled in with dNTPs by the Klenow polymerase and digested with *Avr*II. The resultant products were ligated into pBluescript SK$^+$ then cut with *Xba*I and *Eco*RV. Constructs were subsequently cut with N*ot*I, filled in, and religated. Subclones were designated pSK-CKIα and pSK-CKIαL, having the complete coding sequences of CKIα or αL in-frame relative to the α complementation segment of β-

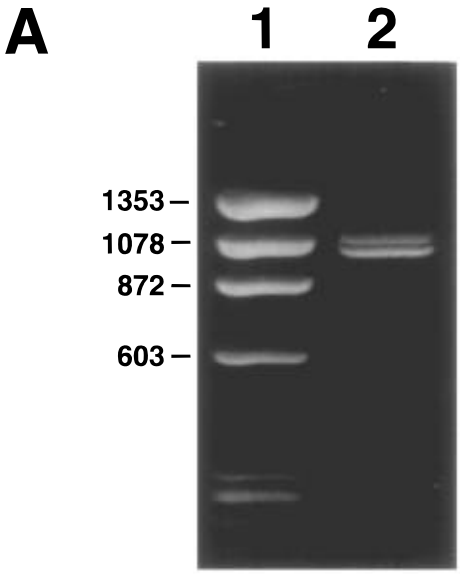
galactosidase. Proteins produced from pSK-CKIα and pSK-CKIαL contain 30 amino acids of the extreme N terminus of the α-complementation fragment of β-galactosidase at their N termini translated from vector sequences. *E. coli* strain DH5α was transformed with either pSK-CKIα or pSK-CKIαL. The transformants were grown up in Luria Broth plus ampicillin (100 μg/mL) at 37 °C to an OD_{600} of 0.2−0.3, induced with IPTG (0.4 mM) and grown for and additional 1−1.5 h at 30 °C. Extracts from the transformed cells were prepared by sonication and used for activity assays and immunological analysis. For the construction of glutathione *S*-transferase (GST) fusion proteins containing CKIα isoforms, rat brain CKIα and CKIαL were subcloned into pGEX-2T (Pharmacia) using the *Sma*I and *Eco*RI restriction sites. Expression and purification of GST−CKIα and GST−CKIαL fusion proteins were performed essentially as described by Smith and Johnson (1988).

Protein Kinase Assays. CKI activity was determined in a 50 μL assay containing 50 mM Tris-HCl, (pH 7.5), 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (30 Ci/mol). For substrate identification, substrates and their corresponding concentrations are indicated in Table 1. Concentrations of substrates for the kinetic assays were varied from 1.5 to 12 μM for casein and phosvitin, 20 to 200 μM for glycogen synthase, and 20 to 160 μM for spectrin. For both substrate determination and kinetic evaluation of the kinase isoforms, 30 and 10 ng of GST−CKIα and GST−CKIαL were added to their respective reaction mixtures. Reaction mixtures were incubated at 30 °C for 15 min and terminated by boiling in the presence of SDS sample buffer for 5 min. Proteins were separated by 10% SDS−PAGE. Substrates were excised for scintillation counting. To serve as background determinations for both the substrate and kinetic analyses, blank reactions lacking kinase were also run. For kinetic quantitation, the substrate was varied at 5 times the K_m value for ATP.

Immunoprecipitation and Western Blotting. Immunoprecipitation of cell lysates was performed essentially as described by Harlow and Lane (1988). Protein concentrations of the lysates were determined using Bradford's method (Bradford, 1976). In brief, affinity-purified antibodies were mixed with cell lysates and incubated at 4 °C with rotation for 1 h. Immunoprecipitates were isolated using protein A−Sepharose and extensively washed. Proteins in the immunoprecipitates were resolved by 10% SDS−PAGE and transferred to a nitrocellulose membrane. The affinity-purified CKIα antibodies were used for immunoblotting. Immunoreactive bands were visualized by either [^{125}I]protein A- or alkaline phosphatase-mediated color reactions as per the manufacturer's protocols.

RESULTS

Demonstration of Two Isoforms of CKIα in Rat Brain. Due to the availability of various rat neuronal cell lines for functional studies, we decided to clone the isoforms of CKIα from rat brain. PCR amplification of adult male rat brain cDNA was conducted with a forward primer corresponding in part to the 5′-untranslated sequence and the N-terminal coding sequence and a reverse primer corresponding to a portion of both the 3′-untranslated region and C-terminal coding sequence of bovine brain CKIα (see Materials and Methods). Two PCR products of similar size were amplified



B

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Rat    CLESPVGKRKRSMTVS P SQDPSFSGLNQ
       |||||||||||||||||  |||||||||||
Bovine CLESPVGKRKRSMTVS T SQDPSFSGLNQ
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FIGURE 1: There are two distinguishable CKIα transcripts in adult rat brain. (A) Ethidium bromide-stained agarose gel of the PCR-amplified DNAs from adult rat brain cDNA. Lane 1 contains 0.3 μg of molecular size markers (ØX174 RF DNA−*Hae* III digests), and lane 2 contains 20 μL of PCR products. (B) Amino acid sequence comparison of the unique region of rat CKIαL with that of bovine CKIαL from Rowles *et al.* (33). The nucleotide sequence for this 28-amino acid peptide has not been published previously.

to different extents from rat brain cDNA (Figure 1A, lane 2). In the sequencing of the PCR products, the smaller, more abundant product was demonstrated to be CKIα, with the larger being CKIαL. The two are identical except for an 84 bp sequence within CKIαL, located between catalytic domains VI and VII (Hanks *et al.*, 1988). The rat brain CKIαL cDNA encodes a 353-amino acid polypeptide of 40.5 kDa. A comparison of the rat and bovine brain CKIα coding sequence reveals 54 nucleotide differences representing 18 codons. Of the 18 different codons, only one [$GAA_{244-246}$ (bovine) → $GGA_{244-246}$ (rat)] results in an amino acid change. The glutamine at position 82 in the bovine protein is a glycine in the rat protein.

Comparison of the amino acid sequence of the insertion region of rat brain CKIαL with that of bovine brain CKIαL reveals an unconserved amino acid change of a proline to a threonine (Figure 1B). Because this was not a conserved change and the sequence was to be used to design a synthetic peptide for antibody production, additional approaches were used to confirm that the sequence was correct and not an error produced by Taq DNA polymerase. First, KlenTaq DNA polymerase was used for PCR due to its higher fidelity (Baranes, 1992). Second, when the PCR products were subcloned, at least five independent subclones were sequenced. Finally, independent partial genomic DNA clones containing the 84 bp exon region were sequenced (see below). Sequences obtained from all these approaches agree with that shown in Figure 1B.

Isoforms of CKIα Are Generated by Alternative Splicing. When adult rat brain cDNA was amplified by PCR with

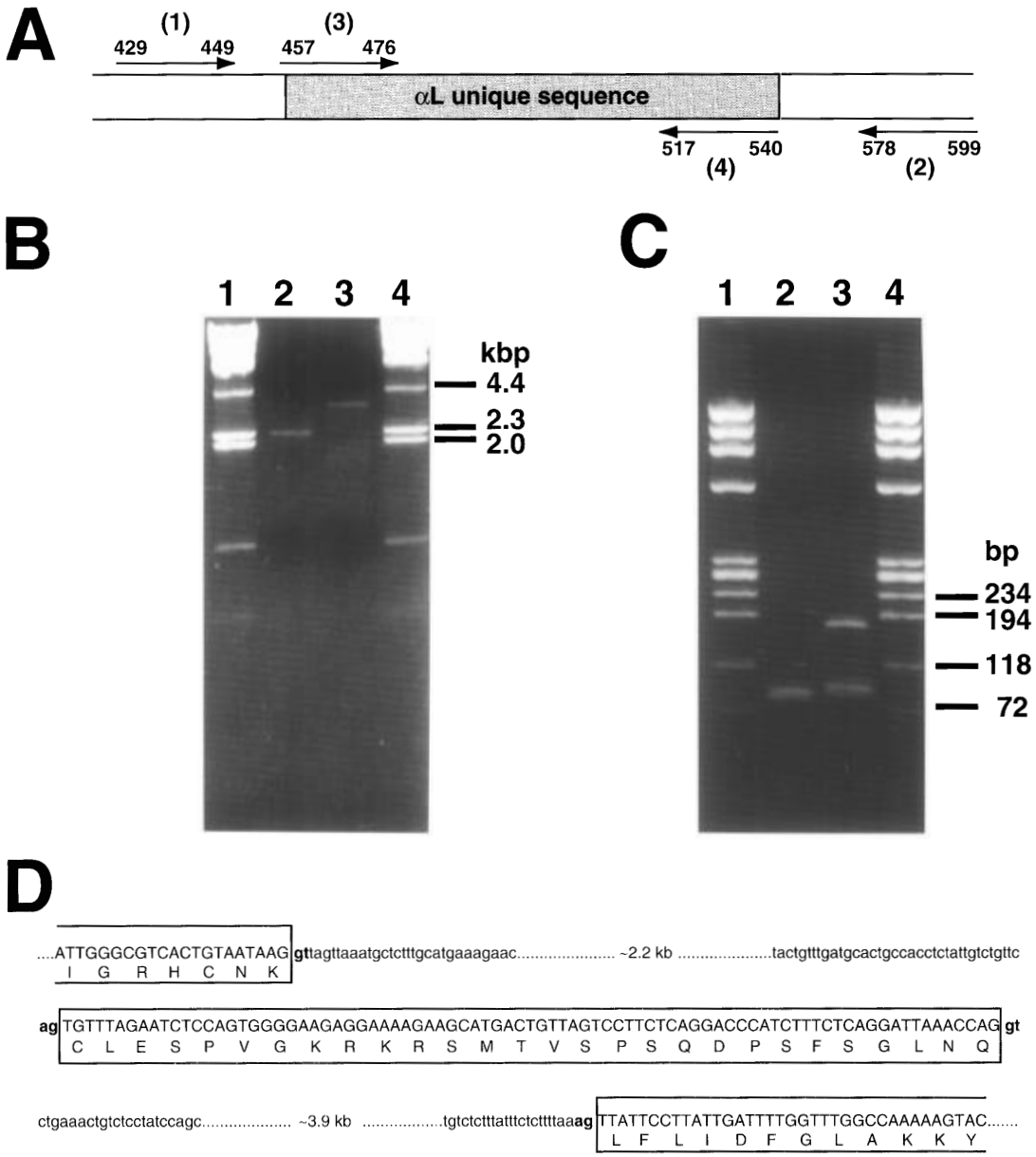

FIGURE 2: PCR of rat genomic DNA and the sequence of the intron/exon boundaries near the CKIαL unique exon region. (A) Scheme of the PCR primers designed to amplify genomic DNA flanking the CKIαL unique sequence. Positions of each primer are numbered, and the sequences of these primers are given in Materials and Methods. (B) The PCR products obtained from rat genomic DNA using primers 1 and 4 (lane 2) and primers 2 and 3 (lane 3); λ DNA−*Hin*dIII digests were used as molecular size markers (lanes 1 and 4). (C) The PCR products obtained from rat brain cDNA using primers 3 and 4 (lane 2) and primers 1 and 2 (lane 3). ØX174 RF DNA−*Hae*III digests were used as molecular size markers (lanes 1 and 4). (D) The PCR products of rat genomic DNA were partially sequenced from both directions. The sequences of the intron/exon boundries are presented.

primers within the unique sequence of CKIαL [primers 3 (forward) and 4 (reverse) shown in Figure 2A], a single product of 84 bp is observed (Figure 2C, lane 2). The size of the DNA fragment was determined using a 6% polyacrylamide/8 M urea gel (Sanger *et al.*, 1977). When PCR was performed with the same cDNA but with primers closely flanking the unique sequence [primers 1 (forward) and 2 (reverse) shown in Figure 2A], two DNA fragments were obtained (Figure 2C, lane 3). Nucleotide sequencing of the PCR products indicated that the upper band matched nucleotides 429−599 of the CKIαL cDNA, and the lower band was identical to the upper band except that it lacked nucleotides 457−540. These results provide further evidence that an insertion of 84 nucleotides results in the appearance of the second transcript from the CKIα gene.

To determine the basis for generation of the two transcripts, genomic DNA was isolated from rat brain and used as a template for PCR. With primers 1 and 2 (Figure 2A), no specific PCR products were detected. However, primers 3 and 4 (Figure 2A) generated a PCR product of the size expected for the αL exon, as depicted in lane 2 of Figure 2C (not shown), indicating that the insert was present within our genomic DNA preparation and detectable by PCR. We suspected that the size of the introns flanking the 84 bp exon could potentially be greater than 5 kbp, which Taq DNA polymerase may not be able to amplify efficiently. Therefore, the combinations of the primers used for PCR of genomic DNA were changed to primers 1 (forward) and 4 (reverse) or primers 3 (forward) and 2 (reverse) to amplify each intron individually. Fragments (2.3 and 4.0 kbp) were

generated using these two pairs of primers, respectively (Figure 2B, lanes 2 and 3, respectively). The fragments were subcloned into M13mp18/19 or pBluescript SK$^+$ and sequenced. Both subclones contained the 84 bp exon of CKIαL at one end. The 2.3 kbp fragment contained a part of a coding sequence upstream of the 84 bp exon, whereas the 4.0 kbp fragment contained a portion of the coding sequence downstream of the 84 bp exon. As shown in Figure 2D, all coding sequences in the partial genomic DNA clones agree completely with the cDNA sequence of rat brain CKIαL. Between these coding sequences are sequences that do not exist in the CKIαL cDNA. Consensus sequences for splicing are found at the intron/exon junctions (Figure 2D). Thus, the CKIα and CKIαL isoforms arise from a common precursor mRNA by alternative splicing.

Expression of Isoforms of CKIα in E. coli. In order to examine the expression of the CKIα isoforms, two polyclonal antibodies were raised against rat CKIαL by immunizing rabbits with either a synthetic peptide (ESPVGKRKRSMTVSPSQDC) conjugated to keyhole limpet hemocyanin (KLH) or a maltose binding protein (MBP) fusion protein containing CKIαL (see Materials and Methods). Both antibodies, when isolated on a peptide−agarose affinity column, recognize CKIαL and not CKIα. A rabbit polyclonal antibody raised against CKIα has been described previously (Brockman *et al.*, 1992) which recognizes both CKIα isoforms (see below).

pSK-CKIα and pSK-CKIαL were generated by subcloning the cDNAs encoding the rat brain CKIα isoforms, in-frame relative to the β-galactosidase α-complementation gene fragment in pBluescript SK+. Extracts of *E. coli* strain DH5α were transformed with pSK-CKIα, pSK-CKIαL, or the pBluescript SK$^+$ vector alone. The resultant lysates were immunoprecipitated with the CKIα and CKIαL antibodies. Immune complexes were separated by 10% SDS−PAGE, transferred to nitrocellulose membrane, and blotted with the CKIα antibody. As illustrated in Figure 3A, four polypeptides of 42, 33, 30, and 21 kDa were precipitated with the CKIα antibody from extracts of the pSK-CKIα transformants (lane 2). The 42 kDa protein is of the size predicted for full-length CKIα expressed from the *lac* promoter. In the case of CKIαL, three polypeptides of 44, 35, and 33 kDa were precipitated with the CKIαL antibody from extracts derived from pSK-CKIαL transformants (Figure 3A, lane 3). The 44 kDa protein is of the size expected for CKIαL expressed from pSK-CKIαL. The number and size of the smaller polypeptides observed for both CKIα and CKIαL varied from preparation to preparation. However, they were always immunoreactive with the CKIα antibody and therefore likely represent proteolytic products or truncated peptides resulting from premature termination of translation. As for the control, there were no immunoreactive proteins immunoprecipitated with CKIα and CKIαL antibodies from extracts of *E. coli* transformed with pBluescript SK$^+$(Figure 3A, lane 1). Casein kinase activities of rat brain CKI isoforms expressed in *E. coli* were then tested using these immunoprecipitated proteins. Both CKIα isoforms isolated from *E. coli* transformants have casein kinase activity, as shown in lanes 5 and 6 of Figure 3A.

Expression Patterns of CKIα Isoforms in PC12 Cells. The calculated mass of rat CKIαL is 40.5 kDa, ~3 kDa larger than the expected size of rat CKIα. This difference is large enough to be resolved by SDS−PAGE. To determine whether CKIαL is expressed *in vivo*, cell lysates of the rat pheochromocytoma cell line PC12 were used to isolate CKIαL by immunoprecipitation with the CKIαL antibody. As a control, a parallel immunoprecipitation experiment was carried out with the CKIα antibody. The resulting precipitates were analyzed by immunoblotting with the CKIα antibody. As illustrated in Figure 3B, the CKIα antibody can precipitate two immunoreactive doublets of approximately 40 and 37 kDa [CKIα and CKIαL (lane 1)] while the CKIαL antibody only precipitates an immunoreactive doublet of around 40 kDa [CKIαL (lane 2)]. Both immunoprecipitates exhibited casein kinase activity (lanes 3 and 4)

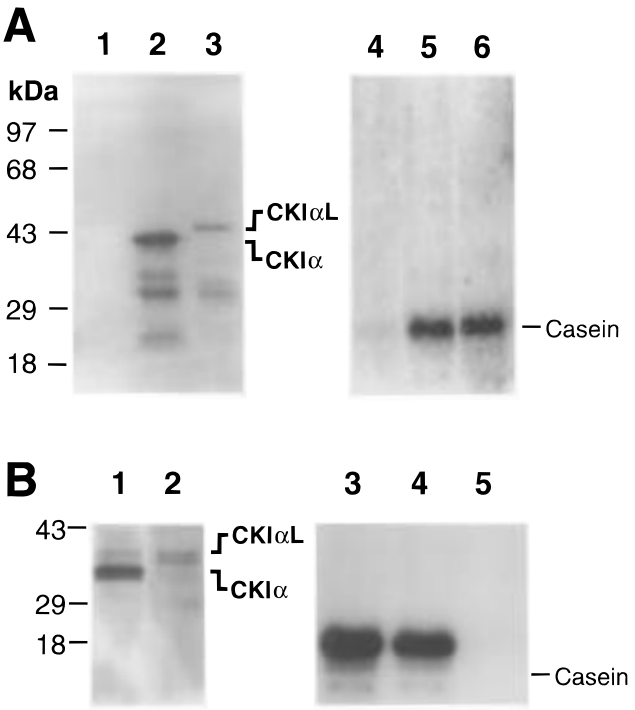

FIGURE 3: Expression of rat brain CKIα and CKIαL in *E. coli* and PC12 cells. (A) The CKIα and CKIαL proteins expressed in *E. coli* and the corresponding casein kinase activity. *E. coli* DH5α was transformed with pBluescript SK$^+$, pSK-CKIα, or pSK-CKIαL. Extracts were prepared from each of the three transformants by sonication. A total of 3.1 mg of soluble protein from each transformant was subjected to immunoprecipitation with the CKIα and CKIαL antibodies. The immunoprecipitates were resuspended in 50 mM Tris-HCl (pH 6.8), resolved by 10% SDS−PAGE, transferred to a nitrocellulose membrane, and blotted with the CKIα antibody. The bound antibodies were detected with [^{125}I]-protein A. The immunoprecipitates were obtained from DH5α/pBluescript SK$^+$ transformants with the CKIα and CKIαL antibodies (lane 1), from DH5α/pSK-CKIα transformants with the CKIα antibody (lane 2), and from DH5α/pSK-CKIαL transformants with CKIαL antibody (lane 3). CKI isoforms were isolated as described above and assayed toward α-casein (as described under Materials and Methods): lane 4, 20 μL of immunoprecipitate obtained from control extracts of DH5α/pBluecript SK$^+$ with both the CKIα and CKIαL antibodies; lane 5, 20 μL of immunoprecipitate obtained from extracts of DH5α/pSK-CKIα with the CKIα antibody; and lane 6, 20 μL of immunoprecipitate obtained from extracts of DH5α/pCKIαL with the CKIαL antibody. (B) PC12 cell lysate (6.5 mg of total protein) was immunoprecipitated with either the CKIα or CKIαL antibody. The resulting immunoprecipitates were resuspended in 100 μl of 50 mM Tris-HCl (pH 6.8), resolved by 10% SDS−PAGE, transferred to a nitrocellulose membrane, and blotted with the CKIα antibody. An alkaline phosphatase-conjugated secondary antibody was employed for the color reaction. Lane 1 contains the proteins immunoprecipitated by the CKIα antibody and lane 2 by the CKIαL antibody. Casein kinase activities of the immunoprecipitated CKIα (lane 3) and CKIαL (lane 4) are indicated. A blank reaction lacking the kinase is shown in lane 5.

Table 1: Substrate Specificity of Recombinant Rat Brain GST−CKIα and GST−CKIαL

substrate [concentration (μM)]	GST−CKIα (pmol min^{-1} mg^{-1})	GST−CKIαL (pmol min^{-1} mg^{-1})
casein [10.5]	9600	28200
SV40 T antigen (101−249) [6.9]	2800	22600
SV40 T antigen (129−249) [6.9]	<20	<30
phosvitin [8.8]	3400	11615
spectrin [1.4]	1600	17500
glycogen synthase [2.3]	6100	3300
histone VIII [20.0]	<20	<30
protamine [75.0]	<20	<30

when compared to a control (lane 5). No immunoreactive proteins were detected when the immunoprecipitations were performed without antibody or, in the case of the αL-specific antibodies, in the presence of the synthetic αL peptide (data not shown). Because the CKIα antibody recognizes both isoforms with equal efficacy, the level of the CKIαL protein is less than 15% of the level of CKIα protein, as determined by using [^{125}I]protein A-mediated immunoblotting.

In addition, when CKIα and CKIαL cDNAs were transiently transfected into PC12 cells in a eukaryotic expression vector, the immunoreactivity and corresponding kinase activity of each isoform was observed to increase on a per milligram PC12 lysate protein basis (data not shown).

Tissue Distribution of CKIα and CKIαL Transcripts and Proteins. The expression of CKIα and CKIαL transcripts in adult rat tissues was examined to establish whether their expression is ubiquitous or tissue-specific. Because the size difference between the two transcripts is small [84 nucleotides in a ≥1.7 kb mRNA (Rowles *et al.*, 1991)], their relative amounts cannot be measured efficiently by Northern blotting. Instead, total RNA was isolated from various tissues of an adult rat, and cDNA was synthesized using AMV reverse transcriptase and an oligo(dT) primer. PCR was then performed on these cDNA pools using a forward primer corresponding to nucleotides 268−289 and a reverse primer corresponding to nucleotides 578−599. As shown in Figure 4A, two DNA fragments of the expected sizes (∼330 and ∼240 bp) were amplified from rat brain, heart, spleen, liver, lung, kidney, and testis total RNA. The resultant PCR products were transferred from the agarose gel to nitrocellulose membrane and hybridized with a ^{32}P-labeled probe corresponding to nucleotides 429−449 of the rat CKIα and CKIαL sequences. Both DNA fragments hybridized with the probe (data not shown). Quantitation of the ^{32}P-labeled DNA fragments revealed that the levels of CKIαL transcripts in rat tissues were 35−55% of those of the CKIα transcripts.

To examine whether these transcripts resulted in the expression of the CKIα isoforms, extracts were prepared from various adult rat tissues and immunoprecipitated with either the CKIα or CKIαL antibody. The resulting immunoprecipitates were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and blotted with the CKIα antibody. The CKIαL and CKIα proteins were detected in all tissues tested (panels B and C of Figure 4, respectively). The CKIαL protein isolated from rat tissues has casein kinase activity (data not shown) and is present at levels lower than those of the CKIα protein (panels B and C of Figure 4), consistent with the analysis of their transcripts. Additional bands immunoprecipitated by the CKIαL antibody from extracts of heart and brain could be some as yet unidentified isoforms of CKIα

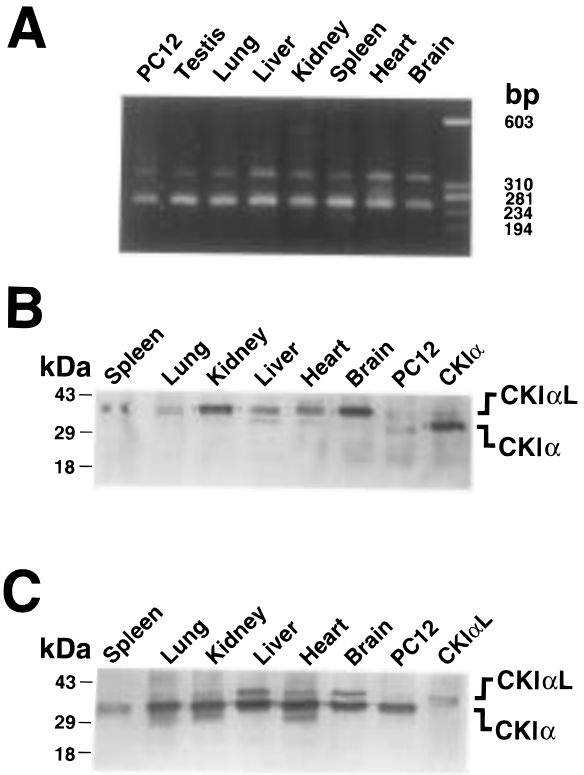

FIGURE 4: Tissue distribution of the CKIα and CKIαL isoforms. (A) Expression of CKIα- and CKIαL-specific transcripts in rat tissues. CKIα and CKIαL transcripts in adult rat tissues were analyzed by RT−PCR using the amplification primers flanking the CKIαL unique exon (as described under Materials and Methods). Lane assignments are as indicated. The final lane contains molecular size markers (ØX174 RF DNA−*Hae*III digests). (B) CKIαL proteins expressed in adult rat tissues. The CKIαL protein was isolated from rat tissues by immunoprecipitation with the CKIαL antibody and blotted with the CKIα antibody. An alkaline phosphatase-conjugated secondary antibody was employed for the color reaction. CKIα isolated from PC12 cells by immunoprecipitation with the CKIα antibody displayed in the final lane (designated CKIα) was used to compare relative mobilities of the two splicing isoforms. (C) CKIα proteins expressed in adult rat tissues. The CKIα protein was isolated from rat tissues by immunoprecipitation with the CKIα antibody and blotted with the CKIα antibody. An alkaline phosphatase-conjugated secondary antibody was employed for the color reaction. Lane assignments are identical to those in panel B with the exception that the final lane contains CKIαL isolated from PC12 cells by immunoprecipitation with the CKIαL antibody to serve as a basis for comparison of its mobility relative to CKIα.

CKIα and CKIαL Are Casein Kinase I Enzymes with Different Substrate Kinetics. To examine whether the CKIα isoforms possess different catalytic properties, the substrate kinetics of the two isoforms were compared using bacterially expressed proteins. The GST system has been employed since in many cases GST fusion proteins possessed native biological activities (Kaelin *et al.*, 1991; Toyoshima & Hunter, 1994; Diekmann *et al.*, 1994) and can be easily purified. The CKIα and CKIαL isoforms were expressed as GST fusion proteins and highly purified (∼90% purity) using glutathione−agarose as described (Smith & Johnson, 1988) (Figure 5, lanes 1 and 2). Their expression was confirmed by Western blotting with isoform-specific antibodies (Figure 5, lanes 3 and 4). Both isoforms could phosphorylate casein, a SV40 large T antigen (101−249)−

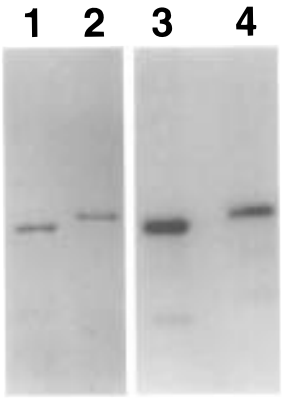

FIGURE 5: Expression and purification of GST−CKIα and GST−CKIαL fusion proteins. Rat brain CKIα and CKIαL cDNAs were subcloned into pGEX-2T in frame and transformed into *E. coli* DH5α, respectively. The transformants were induced with 0.1 mM IPTG, and the overexpressed fusion proteins were purified with glutathione−agarose and analyzed by SDS−PAGE. The recombinant CKIα and CKIαL proteins were visualized by staining with Coomassie blue or transferred to a nitrocellulose membrane and probed with CKIα antibody. Lane 1 contains purified GST−CKIα and lane 2 GST−CKIαL. Lanes 3 and 4 are the corresponding Western blots for the two samples.

Table 2: Kinetic Analysis of GST−CKIα and GST−CKIαL Toward Different Substrates

	GST−CKIα		GST−CKIαL	
	K_m	V_{max}	K_m	V_{max}
casein	13.5	20.7	7.8	48.9
phosvitin	6.4	11.1	59.5	905.7
glycogen synthase	35.7	11.2	63.0	7.4
spectrin	81.9	2.9	11.9	2.6

GST fusion protein, phosvitin, spectrin, and glycogen synthase and have no detectable activities toward histone VIII and protamine (Table 1). GST−CKIα's preferences were as follows: casein > glycogen synthase > phosvitin > SV40 large T antigen (101−249) > spectrin. This order of substrates according to rate very closely approximates those previously reported for CKIα purified from native sources in both order and extent (Tao *et al*., 1980). GST−CKIαL's preferences were as follows: casein > SV40 large T antigen (101−249) > phosvitin > spectrin > glycogen synthase. Using casein phosphorylation as the reference, GST−CKIα is a relatively active glycogen synthase kinase while GST−CKIαL is an effective SV40 large T antigen kinase. Phosphorylation of SV40 large T antigen on serine 123 has been reported previously (Graser *et al.*, 1988). Aside from the differences in substrate kinetics of the two isoforms, the specific activity of GST−CKIαL is higher than that of GST−CKIα for all substrates tested except glycogen synthase (Table 1). Lineweaver−Burke analysis of CKIα and CKIαL was then conducted using casein, phosvitin, glycogen synthase, and spectrin as substrates (Lineweaver & Burke, 1934). The resulting V_{max} and K_m values of CKIα and CKIαL for casein, phosvitin, spectrin, and glycogen synthase are depicted in Table 2. Results indicate that there are distinct kinetic differences between the two enzymes toward these four substrates. While CKIα has a higher affinity for phosvitin and glycogen synthase than CKIαL, its activity toward these substrates and casein is lower, indicating that the αL insert is exerting effects on both substrate affinity and turnover.

DISCUSSION

Some of most striking features of CKI are its diversity of substrates and its multiplicity of subcellular localizations. One explanation is that CKI is actually a large family of genetically distinct isoforms. Some of the inconsistencies in the reported characteristics of the enzyme may be due to different isoforms being examined in a given study. Biological function and regulation of the CKI family cannot therefore be fully established until each of the isoforms has been thoroughly examined.

Two active CKIα isoforms of ∼37 and ∼40 kDa were isolated from rat tissues or PC12 cells by immunoprecipitation with isoform-specific antibodies. Additionally, both CKIα and CKIαL isolated from rat tissues or a PC12 cell line exist as two forms with sizes resolvable by SDS−PAGE. Autophosphorylation is an unlikely explanation of the observed doublets due to the fact that at no time did we observe the presence of these doublets with CKIα or CKIαL derived from *E. coli*. Another splicing isoform (designated CKIα2), with an insert at the C terminus, has also been reported (Fish *et al*., 1995). Conceivably, the doublet that we observe for both CKIα and CKIαL could be due to the insertion of the α2 exon.

Sequencing of rat genomic DNA clones from the CKIα locus revealed that consensus sequences (GT/AG, polypyrimidine tract) for splicing exist at the locations predicted to generate the αL isoform by means of alternative splicing. Although the exon/intron structure of the CKIα has not been fully characterized, the introns flanking the CKIαL unique exon are particularly large at ∼2.2 and ∼3.9 kbp. This observation suggests that the CKIα gene is probably a complex locus capable of directing the synthesis of multiple transcripts. In fact, RT−PCR and immunoprecipitation−Western blotting of liver and kidney extracts revealed higher molecular mass transcripts and proteins detectable with the CKIα cDNA probes and the CKIαL antibody.

The deduced amino acid sequence of the CKIαL unique exon has been examined for similarity with the known sequences in GenBank using the FASTA algorithm (Pearson & Lipman, 1988). No extended regions of similarity were found when the 28 amino acid peptide was compared to other kinases or proteins.

Both CKIα and CKIαL are transcribed and translated in the brain, heart, lung, liver, kidney, spleen, testis, and PC12 cells, as determined by RT−PCR and immunoprecipitation−Western blotting. Because the RT−PCR reactions were carried out with the same pair of amplification primers and RNA preparations, the relative levels of CKIα and CKIαL transcripts can be compared with a reasonable level of confidence. The level of CKIα transcripts is about 2−3-fold more than that of CKIαL in different tissues, suggesting that the CKIαL exon is spliced out from ∼70% of the total CKIα transcripts. These findings suggest that the expression of CKIα and CKIαL can be regulated at the level of alternative splicing. However, the level of CKIαL protein is about 15% of that of the CKIα protein. These results suggest that the expression of CKIαL could be regulated at the post-transcriptional level as well. So far, we do not know whether expression of CKIαL is limited to certain regions of the brain or other tissues.

A number of serine/threonine protein kinases have now been crystallized, including two CKI isoforms (Morgan *et*

al., 1994; Xu *et al.*, 1995; Longenecker *et al.*, 1996). Interestingly, all appear to share common structural elements. Basically, these kinases consist of two lobes joined by a hinge region. The gap between the two lobes serves as the catalytic cleft. Orientation of the lobes relative to one another therefore affects the topology of the catalytic cleft affecting both substrate binding and turnover. The orientation of the lobes is dictated by the conformation of the hinge region. Recently, the crystal structures for a *Schizosaccharomyces pombe* CKI isoform Cki1 and the rat CKIδ isoform have been described (Xu *et al.*, 1995; Longenecker *et al.*, 1996). They too exhibit this bilobal structure (Xu *et al.*, 1995; Longenecker *et al.*, 1996). Because of the sequence conservation among the CKI family members, their structures likely reflect the structures of other isoforms as well (Longenecker *et al.*, 1996). On the basis of this structural evidence, the αL insert would be situated on the surface of the protein within loop L-78 immediately before β8 in a region between the two lobes on the backside of the molecule relative to the active site (Xu *et al.*, 1995; Longenecker *et al.*, 1996). It is possible that the αL insert could affect the flexibility of the kinase, thereby altering the conformation of the catalytic cleft. Therefore, its physical position could conceivably alter both the way in which the kinase interacts with substrate and the turnover rate (Hanks *et al.*, 1988). In addition, the strong positive charge cluster KRKR within the insert could enhance or detract binding of substrate.

Alteration of substrate binding and turnover rate are borne out in these studies. GST−CKIαL exhibits a lower K_m for casein and spectrin and a higher K_m for glycogen synthase and phosvitin. As discussed above, this insert is also positioned to have an effect on the overall rate at which CKI phosphorylates substrate. Kinetic analyses of casein, phosvitin, spectrin, and glycogen synthase indicate that the αL insert enhances the turnover rate toward casein, phosvitin, and spectrin and inhibits the kinase toward casein. Therefore, the sum of the αL insert's affect on both affinity and activity toward substrate can result in substantially different rates of phosphorylation, including potentially relevant *in vivo* substrates such as SV40 large T antigen.

While these kinetic studies were conducted with recombinant GST fusions of the two isoforms exhibiting noticeably lower specific activity than their native counterparts, the relative rates with which they phosphorylate the substrates in Table 1 very closely approximate those reported for CKI purified from native sources (Tao *et al.*, 1980). In addition, these GST fusions are identical to one another in construction, expression, and purification. Any observed differences are therefore due to the presence or absence of the αL insert.

Alternative splicing resulting in different kinases is not unique to CKI. The FGF receptor gene family may encode as many as 96 different receptors, mostly through alternative splicing of extracellular sequences (Dionne *et al.*, 1991). Some members of the activin family of receptor serine/threonine kinases are generated by alternative splicing within the cytoplasmic juxtamembrane region (Attisano *et al.*, 1992). Protein kinase C isoforms β_I and β_{II} also result from alternative splicing (Ono *et al.*, 1987). However, none of these kinases contains inserts within their catalytic domains. Only the TrkC tyrosine kinase receptor is known to possess an insert of either 14 (TrkC k2) or 25 (TrkC k3) amino acid residues between tyrosine kinase subdomains VII and VIII. These isoforms perform distinct biological functions and possess different substrate specificities (Lamballe *et al.*, 1993). To our knowledge, with the exception of CKIαL, no alternatively spliced isoforms of serine/threonine kinases containing inserts in the catalytic domain have been described.

We have demonstrated for the first time that an active CKIαL protein exists in rat brain and other tissues. CKIαL can be separated from CKIα by immunoprecipitation with the purified CKIαL antibody. With one exception, both the immunopurified and recombinant CKIαL protein appear to have higher intrinsic kinase activities than do the CKIα isoforms. Taken together, the CKIα and CKIαL isoforms are ubiquitous kinases with different substrate affinities and specific activities. They are generated by alternative splicing and exist in quite different amounts within a given cell. The expression of CKIα and CKIαL may be regulated at different levels, including at the level of alternative splicing, mRNA turnover, and translation.

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