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Biochemical and Cellular Characteristics of the Four Splice Variants of Protein Kinase CK1α From Zebrafish (*Danio Rerio*)

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Abstract Protein kinase CK1 (previously known as casein kinase I) conforms to a subgroup of the great protein kinase family found in eukaryotic organisms. The CK1 subgroup of vertebrates contains seven members known as α, β, γ1, γ2, γ3, δ, and ε. The CK1α gene can generate four variants (CK1α, CK1αS, CK1αL, and CK1αLS) through alternate splicing, characterized by the presence or absence of two additional coding sequences. Exon ''L'' encodes a 28-amino acid stretch that is inserted after lysine 152, in the center of the catalytic domain. The ''S'' insert encodes 12 amino acid residues and is located close to the carboxyl terminus of the protein. This work reports some biochemical and cellular properties of the four CK1α variants found to be expressed in zebrafish (*Danio rerio*). The results obtained indicate that the presence of the ''L'' insert affects several biochemical properties of CK1α: (a) it increases the apparent Km for ATP twofold, from ~30 to ~60 μM; (b) it decreases the sensitivity to the CKI-7 inhibitor, raising the I_{50} values from 113 to ~230 μM; (c) it greatly decreases the heat stability of the enzyme at 40°C. In addition, the insertion of the ''L'' fragment exerts very important effects on some cellular properties of the enzyme. CK1αL concentrates in the cell nucleus, excluding nucleoli, while the CK1α variant is predominantly cytoplasmic, although some presence is observed in the nucleus. This finding supports the thesis that the basic-rich region found in the ''L'' insert acts as a nuclear localization signal. The ''L'' insert-containing variant was also found to be more rapidly degraded (half-life of 100 min) than the CK1α variant (half-life of 400 min) in transfected Cos-7 cells. J. Cell. Biochem. 86: 805–814, 2002.

Key words: casein kinase 1; alternative splicing; nuclear localization signal; heat stability; CK1-7 inhibition; apparent Km for ATP; protein turnover

Protein kinase casein kinase 1 (CK1) conforms to a subgroup of the great protein kinase family of enzymes that is present in all eukaryotes [Hanks and Hunter, 1995]. CK1 is mainly a ser/thr kinase although it can phosphorylate certain tyrosine residues at low efficiency. In mammals there are seven different genes that code for CK1 isoforms: α, β, γ1, γ2, γ3, δ, and ε [Reviewed in Gross and Anderson, 1998]. These isoforms are 50% identical in their catalytic domain but differ greatly at the carboxyl end. The α and β isoforms are the smallest (37 and 39 kDa), whereas the remaining five isoforms vary from 45 to 49 kDa and have extended carboxyl regions that include some autophosphorylation sites that can regulate their activity [Rowles et al., 1991; Graves et al., 1993; Fish et al., 1995; Cegielska et al., 1998]. CK1 isoforms have been involved in a variety of physiological and cellular processes of great interest such as circadian rhythm [Kloss et al., 2001], Wnt signaling [Peters et al., 1999; Liu et al., 2002], neurotransmission [Liu et al., 2001], DNA repair [Ho et al., 1997], vesicle trafficking [Murakami et al., 1999], and T-cell regulation [Zhu et al., 1998; Marin et al., 2002]. There are also a large number of other substrates that have been shown to be phosphorylated by CK1.

Studies on the amino acid sequence requirements for phosphorylation of peptides by CK1 isoforms demonstrate that these enzymes have a high preference for substrates that contain

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phosphoserine or phosphothreonine in position −3 regarding the acceptor serine or threonine (pS/pTXXS*/T*, where pS and pT denote phosphorylated serine and threonine, S*/T* the phosphorylatable serine or threonine and X, any amino acid) [Flotow et al., 1990; Meggio et al., 1991, 1992]. This property is the basis for the participation of CK1 in hierarchical or "primed" phosphorylation in which a substrate is first phosphorylated by another kinase before it can be acted upon by CK1 [Flotow and Roach, 1989; Flotow et al., 1990]. Other notable examples of kinases that can be primed to phosphorylate physiological substrates are CK2 [Allende and Allende, 1995] and GSK-3 [Frame and Cohen, 2001; Harwood, 2001]. It is, therefore, interesting that CK1 and these other kinases may be able to prime each other [Liu et al., 2002]. However, CK1 does not only phosphorylate previously phosphorylated peptides. Detailed analysis of protein substrates and model peptides has demonstrated that the presence of acidic amino acid residues at position −3 from the target serine or threonine also defines a consensus sequence for this family of enzymes (D/EXXS*/T*) and that the presence of other acidic residues surrounding the −3 position also increases the efficiency of the target sequence [Flotow and Roach, 1991; Pulgar et al., 1999].

The CK1α isoform has been found to contain several splice variants in rat [Zhang et al., 1996], chicken [Green and Bennet, 1998], and human [Tapia et al., 1994; Fish et al., 1995; Yong et al., 2000]. These variants, which result in the incorporation of two short inserts, can generate four different CK1α structures. The longer insert (28 aa) which is called L, is positioned in the middle of the catalytic domain while the shorter variable insert (S), is located very close to the carboxyl end. The four variants, therefore, are CK1α, CK1αL, CK1αS, and CK1αLS (Fig. 1A).

In this communication, we present the cloning and sequence of the four splice variants of zebrafish (*Danio rerio*) CK1α and studies on their biochemical and cellular characteristics. The variants containing the L insertion were found to be more sensitive to temperature inactivation and to have an apparent Km for ATP significantly higher than the variants that lack this insertion. Similarly, the CK1α forms containing the L sequence were less sensitive to CKI-7, a specific inhibitor of CK1. The most dramatic difference of these variants, however, was their subcellular localization, since a variant containing the L insert, CK1αL, was predominantly concentrated in the nucleus, while the CK1α variant, which lacks this insertion, was predominantly cytoplasmic. Metabolic labeling of transfected CK1α splicing variants demonstrated that the presence of the L insert caused the enzyme to be more rapidly degraded compared to the variant lacking this insert.

MATERIALS AND METHODS

[^{32}P-γ]ATP, [^{32}P-α]dATP, [^{32}P-α]dCTP, and Easytag^{TM} EXPRE^{35}S^{35}S Protein Labeling Mix [^{35}S] were obtained from New England Nuclear. Restriction enzymes were from Promega. Oligonucleotides were synthesized at Oligopéptido, Faculty of Medicine, Universidad de Chile. Anti-HA monoclonal antibody (F-7) was from Santa Cruz and anti-mouse IgG conjugated to fluorescein isothicyanate (FITC) was from DAKO, as well as the fluorescent mounting medium. DMEM (complete and without L-Met/L-Cys) was from Sigma. Ni^{++}-nitrilotriacetate (NTA)-agarose was obtained from Qiagen and FuGene^{TM} transfection reagent was from Roche Biochemicals. BCA Protein Kit was from Pierce. The dsDNA Cycle Sequencing System was obtained from GIBCO BRL Life technologies (actually Invitrogen).

Cloning and Expression of Zebrafish CK1α Splicing Variants

A 24–48 h zebrafish embryo cDNA library in λZAP (Stratagene) was screened using a ^{32}P-labeled 159 bp fragment derived from a *Xenopus laevis* CK1α cDNA clone, obtained previously in our laboratory [Pulgar et al., 1996]. Four positive clones, two of which encoded CK1α and the other two, CK1αS, were obtained. Primers, P1 (5′GCCGTCACTGTAATAAG3′) and P2 (5′GACCAAAATCGATAAGGA3′), flanking the L sequence insertion site, were designed based on the nucleotide sequence of the clones isolated from the library, and used in RT-PCR experiments to amplify the L exon. The corresponding fragment obtained was sequenced and used as a probe to screen the zebrafish library. One clone was obtained that was truncated at the 5′ and 3′ ends and which encoded a fragment of a CK1α variant that contained the L insert. In order to obtain complete cDNAs for CK1αL and CK1αLS, this clone was digested with *Bgl*II and *Sal*I and ligated into CK1α and CK1αS clones previously digested with the same enzymes.

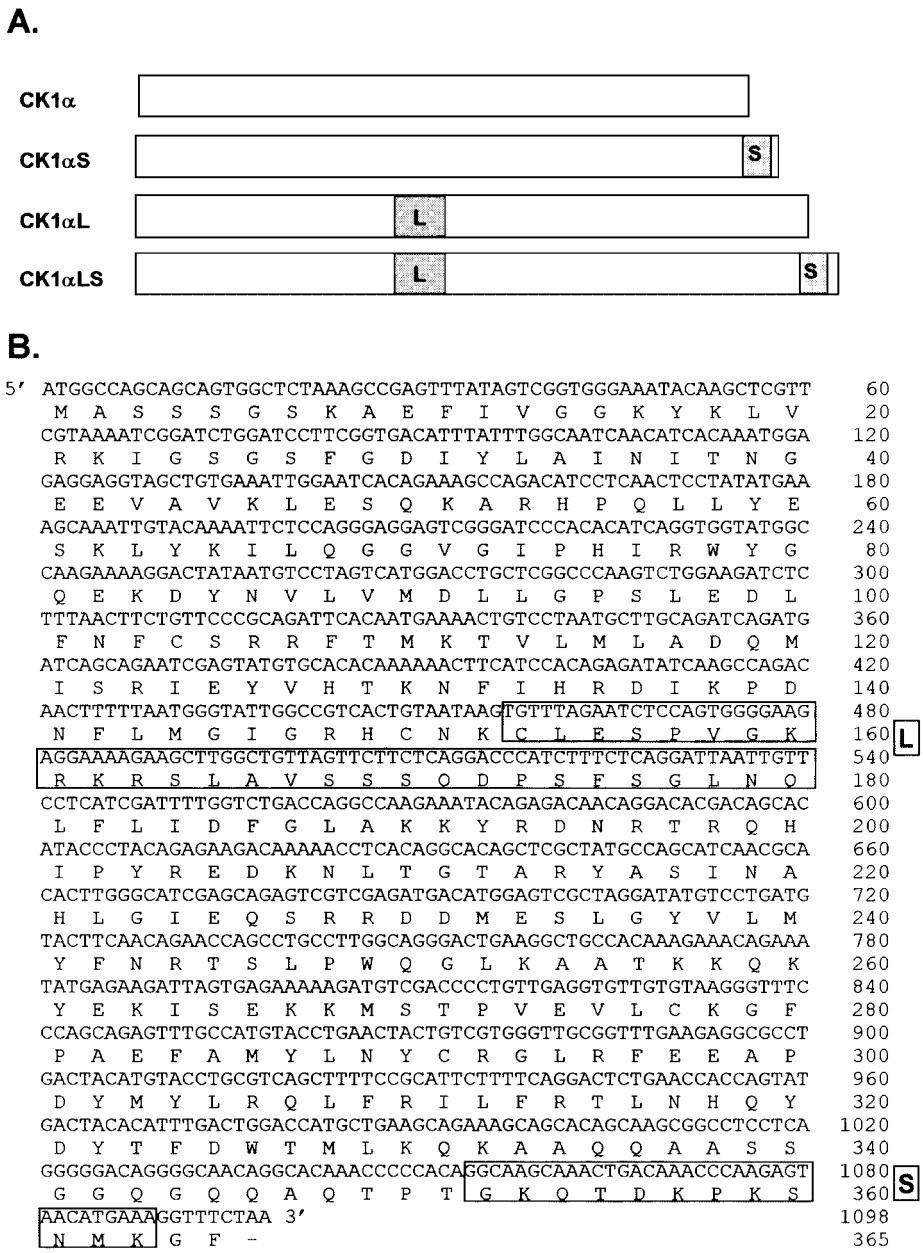

Fig. 1. The four variants of zebrafish CK1α. (**A**) Scheme depicting the overall structure of the four splicing variants of zebrafish CK1α: α, αS, αL, and αLS. (**B**) Coding region of zebrafish CK1αLS cDNA. Deduced amino acid sequence is shown below the nucleotide sequence. Nucleotide and amino acid sequence of L and S inserts are boxed.

To obtain bacterial expression, the coding sequence of the four splicing variants was subcloned into pT7.7-H6 and introduced into *Escherichia coli* BL21(DE3) cells by electroporation. Cells were grown at 37°C to an absorbance at 600 nm of 1.0. Expression of 6His-tagged proteins was induced overnight at 15°C in the presence of 0.2 mM isopropyl-β-D-thiogalactoside. Afterwards, cells were pelleted at 3,000*g* for 20 min at 4°C and cell pellets were resuspended in buffer A (50 mM HEPES, pH 8.0, 500 mM NaCl) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml each of leupeptin, aprotinin, pepstatin A, and antipain. Lysozyme (1 mg/ml) was added and cells were lysed for 1 h on ice. Lysates were sonicated and centrifuged at 39,000*g* for 30 min at 4°C. Supernatants were combined with Ni^{++}-NTA-agarose and incubated on a rocking platform for 1 h at 4°C.

Suspensions were mounted on 5-ml columns and resin was washed with 40 vol buffer B (50 mM HEPES, pH 7.5, 0.2 M NaCl, 1% Triton X-100), followed by 20 vol buffer B with 10 mM imidazole. Recombinant proteins were batch-eluted with 10 vol buffer B containing 200 mM imidazole.

Genomic Structure of the Variable Inserts

The overall genomic structure of the zebrafish CK1α gene has been elucidated and will be published elsewhere (Antonelli et al., in preparation). The positioning of the inserts introduced by alternative splicing was determined by PCR amplification of zebrafish genomic DNA. The sequences of exon–intron boundaries were determined by comparing the sequence of genomic DNA and the cDNA sequence of the zebrafish CK1α gene. Genomic DNA was isolated from adult zebrafish caudal fin as described inWesterfield [1993]. Inserts L and S were amplified by PCR using specific primers obtained from the CK1α cDNA sequence (insert L: 5′TCGAGTATGTGCACACAAAAA3′ and 5′GCCTGTGAGGTTTTTGTCTTC3′; insert S: 5′TTGACTGGACCATGCTGAAGCAGA3′ and 5′ACAGGAAAAAGGTTGCCCACAG3′).

RT-PCR Amplification of mRNA of CK1α Splice Variants

Total RNA was isolated from adult zebrafish using the TriZol™ Reagent (Invitrogen). One microgram RNA was reverse-transcribed with Superscript™ reverse transcriptase (Invitrogen), using an oligo(dT) primer and cDNAs were amplified by PCR using P1 (described above) and P3 (5′TGGTGAATTTGATCCTAG3′) primers. Reactions were analyzed on a 3% agarose gel stained with ethidium bromide.

Kinase Assays and Determination of Apparent Km Values

Standard reaction mixes contained 5–20 U of enzyme (1 U was defined as the amount of enzyme required to transfer 1 pmole/min of phosphate from ATP to β-casein), 50 μM [^{32}P-γ]ATP (1,000–2,000 cpm/pmole), and 5 mg/ml β-casein in kinase buffer, consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, and 10 mM $MgCl_2$, in a total volume of 30 μl. The reaction was started by the addition of ATP and stopped after 10 min at 37°C, by adsorption onto phosphocellulose paper. Papers were washed three times for 10 min in 75 mM phosphoric acid, dried, and radioactivity determined by scintillation. When used, inhibitors were added before ATP.

Transfection of Cultured Cos-7 Cells and Immunofluoresence

Cos-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) on coverslips in 60 mm plates at 37°C and 5% CO_2. At 50–90% confluency, cells were transfected with 4 μg DNA from CK1α or CK1αL constructs in pCEFL-HA vector, using the FuGene transfection reagent, according to manufacturer's directions. At 48 h post-transfection, cells attached to coverslips were washed three times with PBS and fixed in 3.7% *p*-formaldehyde for 5 min. After three PBS washes, cells were permeabilized for 5 min in methanol at −20°C, washed again and incubated 10 min in PBS containing 1% skim milk. Cells were then incubated in a 1:25 dilution of monoclonal anti-HA in PBS for 45 min at 37°C. After three PBS washes, cells were incubated in a 1:50 dilution in PBS of anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) for 45 min at 37°C. Cells were washed three times in PBS, incubated 3 min in 5 μg/ml propidium iodide (PI), washed again, and mounted onto glass slides in fluorescent mounting medium. Fluorescent images were obtained on a Zeiss Axiophot confocal microscope.

Pulse-Chase Experiments

Cos-7 cells were transfected as described above, without the coverslips. At 32 h post-transfection, cells were washed thoroughly with sterile PBS and DMEM lacking L-methionine and L-cysteine, supplemented with 10% FBS and 0.1 mCi/ml [^{35}S]methionine/cysteine was added. Cells were labeled 16 h at 37°C, after which they were washed thoroughly with sterile PBS. Medium was replaced by cold DMEM/FBS containing excess non-radioactive L-methionine and L-cysteine and samples were collected by scraping at 0, 30, 60, 110, and 300 min. Detached cells were washed in PBS and incubated for 30 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium vanadate, 1 mM PMSF) containing 2 μg/ml each of leupeptin, aprotinin, pepstatin A, and antipain. After pelleting the lysates at 14,000*g* in microfuge, protein concentration was determined with the use of the BCA Protein Assay Kit (Pierce). A total of 200 μg protein from each lysate was immunoprecipi-

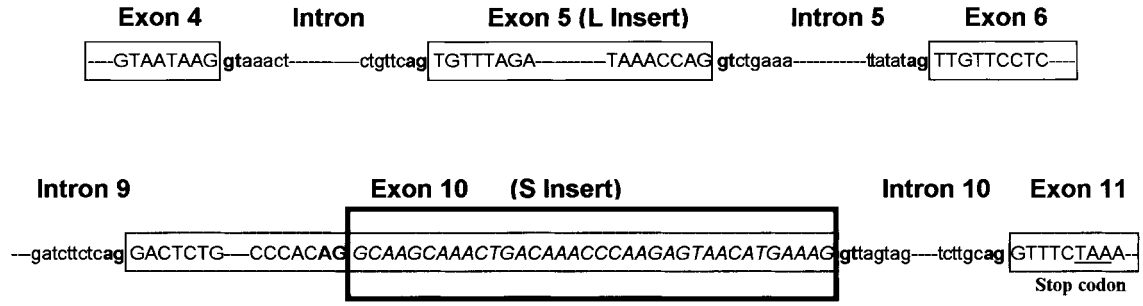

Fig. 2. Scheme of the variant splice sites used to generate the L and S inserts of CK1α. The exon sequence is represented by uppercase letters and the intron sequence by lower case letters. The canonical consensus splice sites, ag at the 3′ splice site and gt at the 5′ splice site, are in boldface. Exon sequences are in boxes. "S" insert is indicated by boldbox.

tated with 2 μl of monoclonal anti-HA antibody for 1 h at 4°C on a rocking platform. Twenty microliters of 50% protein G-agarose was added and incubation proceeded overnight. Immunoprecipitates were washed five times with lysis buffer and 30 μg of total protein was analyzed on a 10% denaturing SDS–PAGE gel. The gel was dried and exposed for 5 days at −70°C to Kodak BIOMAX MS film. Relative amounts of labeled protein were determined by densitometric analysis, using a stable protein band as an internal standard.

Sequencing

The cDNA clones and other constructs were sequenced by PCR with the use of the dsDNA Cycle Sequencing System, based on the Sanger et al. [1997] method. The sequence of the CK1αS, CK1αL, and CK1αLS cDNA clones can be accessed at GenBank with the accession numbers AY116143, AF516329, and AF516328, respectively.

RESULTS

Four Splice Variants of CK1α in Zebrafish

Using probes derived from the *X. laevis* CK1α, previously cloned in our laboratory, [Pulgar et al., 1996], a cDNA library of zebrafish embryos was screened and clones encoding the four isoforms were isolated or constructed as described in Materials and Methods.

Figure 1B shows the nucleotide sequence of the coding region of the cDNA coding for the CK1αLS isoform of zebrafish (*D. rerio*). In the same figure, the corresponding amino acid sequence for the CK1αLS protein is indicated. The stretches corresponding to the L and S exons are boxed. In Figure 1A, we see the schemes corresponding to the four isoforms of zebra fish CK1α, CK1αL, CK1αS, and CK1αLS. The total genomic structure of the CK1α gene of *D. rerio* was determined and found to contain 11 exons interrupted by 10 introns and to span 25 kb [Antonelli et al., in preparation]. The exon containing the L insert corresponds to exon 5. On the other hand, the S insert (amino acids 352–363) is encoded by the C-terminal portion of exon 10, which codes for amino acids 315–363 (Fig. 1). In CK1α and CK1αL, a splice site contained in nucleotides 1,053-1,054 of this exon is used to splice out the S insert-encoding sequence, together with intron 10 and to join amino acid 351 to the carboxyl-terminal GF sequence present in the last exon (Exon 11). Figure 2 gives the corresponding donor and acceptor sequences of the alternatively spliced variants.

Figure 3 shows an RT-PCR amplification of total RNA obtained from adult zebrafish in which primers were designed to test the presence of mRNA representing the four splice variants of CK1α. It can be seen that the most

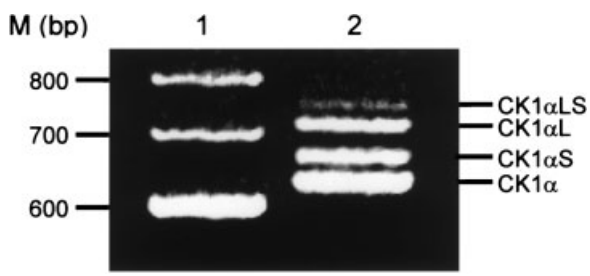

Fig. 3. RT-PCR amplification of adult zebrafish total RNA. One microgram of RNA was reverse-transcribed using oligo(dT) primer and the resulting cDNA was amplified by PCR using the primers P1 and P3 described in Materials and Methods. The amplification products were analyzed on a 3% agarose gel. In **lane 1**, the position of 100 bp DNA ladder standards is shown, with respective sizes (M). **Lane 2** depicts the amplification products of the RT-PCR.

abundant variant is CK1α, which lacks both of the alternative exons. The forms that contain one of the two inserts, αS and αL, exhibit a similar level of expression, while the mRNA for the longest form, CK1αLS, is much less abundant compared to the others.

Biochemical Characterization of the Four Splice Variants of CK1α

Apparent Km for substrates. The question arises as to whether the insertion of L or S can affect the biochemical properties of the CK1α enzyme. In order to address this issue, the cDNAs corresponding to the four splice variants of CK1α were introduced in the vector pT7-7H6 and expressed in *E. coli* BL-21 with an N-terminal $(His)_6$ tag in order to facilitate their purification on Ni^{++}-NTA-agarose columns.

The apparent Km values of substrates for the four CK1α splice variants are shown in Table I. There are no significant differences in the apparent Kms of the variants for protein or peptide substrates. However, a consistent twofold increase is observed in the apparent Km for ATP by the two forms that contain the "L" insert as compared to those variants lacking this sequence. The doubling of the Km values for ATP by the variants containing the "L" insert was reproducibly observed in three separate experiments.

Response to Inhibitors

Copolyglutamic and tyrosine (poly[Glu^{80}:Tyr^{20}]) are synthetic peptides that can act as strong inhibitors of CK1 and CK2 [Tellez et al., 1990; Pulgar et al., 1996]. The inhibition of the four variants was essentially similar with poly [Glu^{80}:Tyr^{20}], with a slightly higher sensitivity displayed by the L variants (not shown). However, the variants containing the L insert are significantly less sensitive to the inhibitor CKI-7 than the shorter forms as can be seen in Figure 4. The I_{50} of CKI-7 for CK1α and CK1αS is 113 μM, while the I_{50} values for CK1αL and CK1αLS are 236 and 224 μM, respectively.

TABLE I. Apparent Km Values of Zebrafish CK1α Splicing Variants Towards Different Substrates

	CK1α	CK1αS	CK1αL	CK1αLS
β-Casein (mg/ml)	1.23	1.03	1.58	1.23
PPI 2 peptide[a] (μM)	228	235	237	227
Phosvitin (mg/ml)	3.21	2.75	2.48	2.42
ATP (μM)	33	30	69	55

[a]RRKDLHDDEEDEAMSITA.

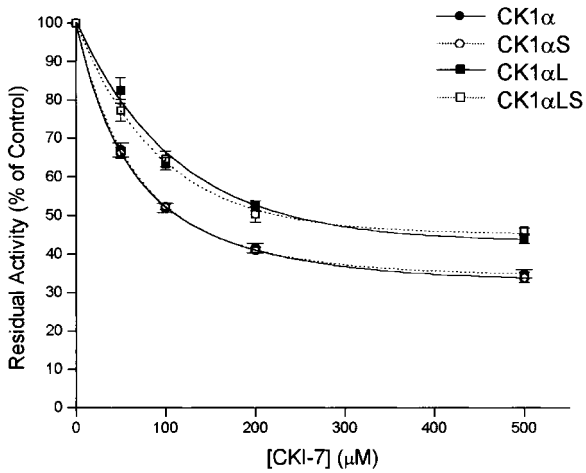

Fig. 4. Inhibition of CK1α splicing variants by CKI-7. Initial velocity was measured in triplicate under standard assay conditions and in the presence of increasing concentrations of CKI-7. Each point was normalized against controls containing the corresponding concentration of DMSO.

CKI-7 is a well characterized specific inhibitor of CK1 that is known to act as an analogue of ATP [Chijiwa et al., 1989].

Thermal Stability

The inactivation by incubation at 40°C of the four variants during different periods of time is shown in Figure 5. It can be seen that the variants with the L insert are inactivated more rapidly, reaching 50% activity after ~2 min, while the variants that lack this insert take about 8 min to reach a similar level of activity.

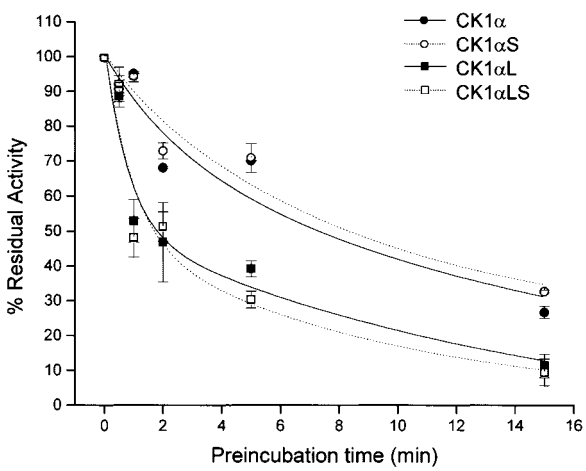

Fig. 5. Thermal stability of CK1α splicing variants at 40°C. Reaction mixes, containing all the reagents but ATP, were incubated for different periods of time at 40°C, after which [^{32}P-γ] ATP was added and the kinase reaction was carried out as described in Materials and Methods, at 30°C for 10 min. Determinations were carried out in triplicate.

Cellular Localization

The splice variants CK1α and CK1αL were subcloned into the eukaryotic vector pCEFL-HA and transfected into Cos-7 cells. The resulting ectopically expressed proteins contained an N-terminal [HA] tag, which allows their detection by immunofluorescence using anti-HA antibodies. The confocal microscope images shown in Figure 6 demonstrate the results obtained using this approach. A very clear difference is observed between the short form CK1α and the longer CK1αL variant in their cellular localization.

CK1αL is predominantly nuclear, as can be clearly observed by co-localization with the specific nuclear stain (PI), while a small portion of the protein is distributed in the cytoplasm. The CK1α variant is present throughout the cell predominantly in the cytoplasm and, in some cases, this variant seems to be excluded from the nucleus. It is very clear also that both variants are absolutely excluded from nucleoli.

Turnover of Labeled Variants

There is currently no information available on the turnover of CK1α in living cells. In order to study this parameter, Cos-7 cells transfected with HA-CK1α and HA-CK1αL were metabolically labeled with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine, followed by a chase with unlabeled amino acids. Radioactivity associated with the variants was analyzed by SDS–PAGE and autoradiography of immunoprecipitates of lysates from cells submitted to different chase periods. The results, presented in Figure 7, show that the drop in radioactivity of the CK1α variant that lacks the L insert is much slower than the variant containing this sequence. CK1αL is degraded more rapidly, displaying a half-life of 100 min, while CK1α shows a half-life of 400 min.

DISCUSSION

The work presented above has defined the existence of four alternatively spliced variants of CK1α mRNAs in zebrafish (*D. rerio*). The only other species where these same four variants had been previously isolated is chicken (*G. gallus*) [Green and Bennet, 1998]. In bovine and human the existence of the CK1αL variant has been identified [Rowles et al., 1991; Yong

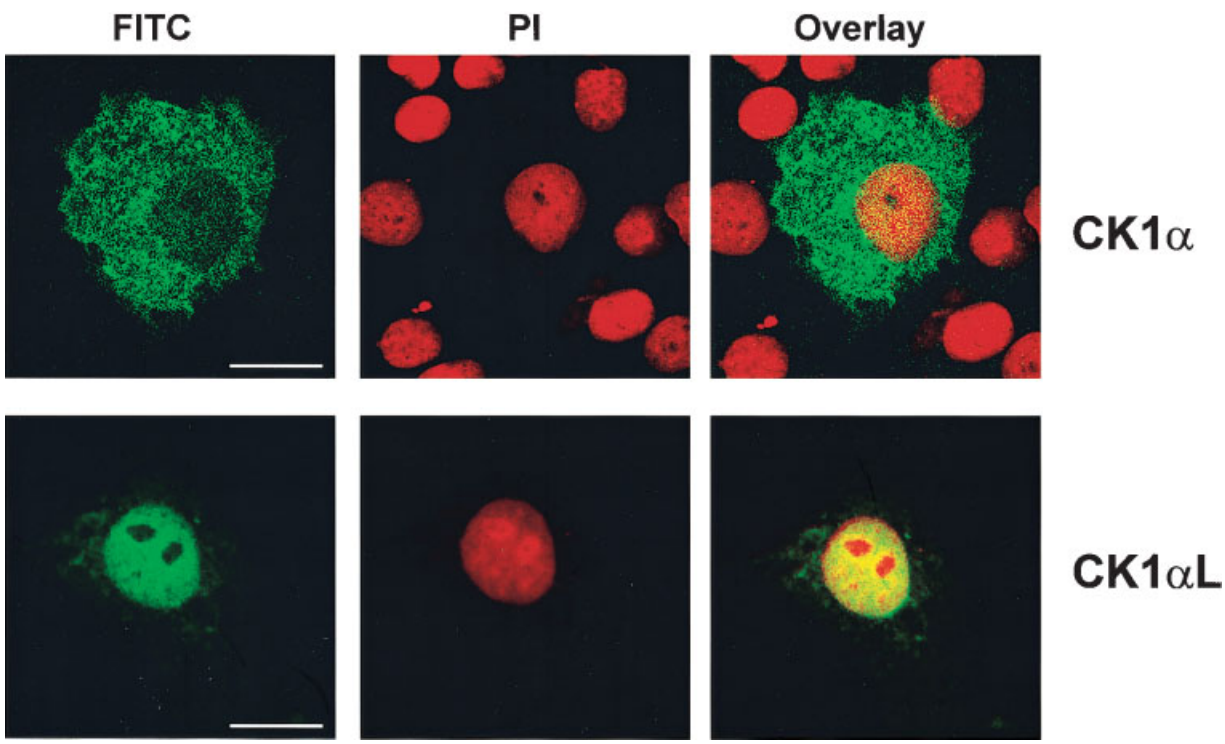

Fig. 6. Immunolocalization of transfected CK1α and CK1αL. Cos-7 cells were transfected with Pcefl-HA constructs containing the CK1α or the CK1αL cDNA and, 48 h post-transfection, cells were fixed and incubated with monoclonal anti-HA, followed by polyclonal anti-mouse IgG conjugated to FITC as described in Materials and Methods. Cells were finally stained with PI, mounted in fluorescent mounting medium and analyzed under a Zeiss Axiophot confocal microscope. The images of FITC and PI fluorescence are shown, together with an overlay of both flourophores. Bars represent 10 μM.

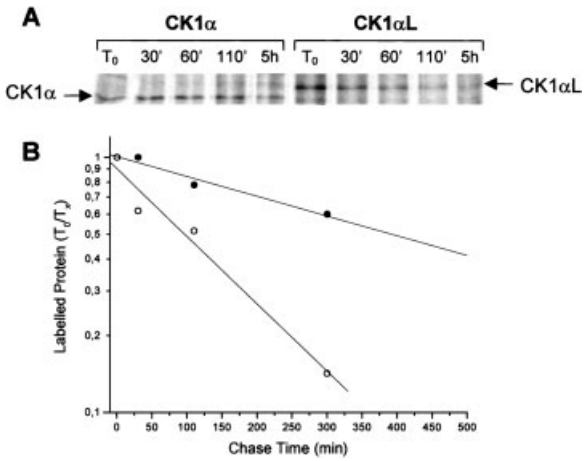

Fig. 7. Determination of CK1α and CK1αL half-lives by pulse-chase labeling of transfected cells. Cos-7 cells transfected with HA-tagged CK1α and CK1αL were metabolically labeled for 16 h with a mixture of [^{35}S]Met and [^{35}S]Cys, after which cells were washed thoroughly and incubated in a medium containing an excess of unlabeled L-methionine and L-cysteine. Samples were collected at the indicated times and processed as described in Materials and Methods. (**A**) Autoradiography of immunoprecipitates of HA-CK1α and HA-CK1αL obtained at T_0 and 30, 60, 110, and 300 min of chase. (**B**) Graphic representation of labeled protein decay, as determined by densitometric analysis. Half-life was 97 min for CK1α L(o) and 400 min for CK1αL.

et al., 2000] and the S insertion has been reported in human [Tapia et al., 1994; Fish et al., 1995]. The data on the human genome localized the CK1α gene to chromosome 5 and ESTs have demonstrated the expression of the four variants.

The cloning of these CK1α variants from zebrafish demonstrates again the extremely high degree of conservation of the sequence of CK1α in vertebrates. Zebrafish CK1α is identical to the enzyme from human, bovine, or *X. laevis* with only one amino acid difference. The "L" insert which is encoded by exon 5 of the zebrafish gene has only three amino acid changes in a total of 28 amino acids, compared to chicken, rat, and human. The "S" insert, which is encoded by a portion of exon 10 of the zebrafish gene, contains a single difference with respect to chicken. It is interesting to note that the infrequent alternate splicing that deletes the "S" part of exon 10 to give rise to CK1α and CK1αL occurs in exactly the same position in the human CK1α gene, which is located on chromosome 5. A study of the boundary sequences between exons and introns in those that are alternatively spliced demonstrates that these conform to the canonical sequences that have been established for splice donor and acceptor sites [Antonelli et al., in preparation].

The recently published human genome data was surprising with regards to the small number of genes in vertebrates as compared to those of what we consider simpler organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*. This finding brought us to the realization of the importance of other mechanisms that contribute to cellular and organismic complexity. One of these mechanisms is alternate splicing of mRNAs, which can generate several different protein products out of one gene. For this reason, it has become important to analyze the different properties that proteins can acquire through alternate splicing. In the case of CK1α, the "L" insert is especially interesting because it is situated within the catalytic kinase domain, between regions VIb and VII, conserved in all kinases, and in close proximity to the "hinge" segment that separates the N-terminal β-sheet domain from the carboxyl α-helical domain. Analysis of hundreds of kinase sequences demonstrates that an insertion of this magnitude in this region is rather unique. On the other hand, the carboxyl extension accomplished through the insertion of "S" is quite common, and various species of CK1 have rather long extensions at this end. In CK1δ and CK1ε, the carboxyl-terminal domains contain autophosphorylation sites that negatively regulate their activity [Graves and Roach, 1995; Cegielska et al., 1998].

Bioinformatic searches of the databanks for possible similarities of the "L" insert sequence to the sequences found in other protein domains yielded some suggestive results. The most obvious one is the possibility that the basic amino acid cluster spanning residues 8–11 of the insert (residues 160–163 in the protein) may act as a nuclear localization signal. As it was demonstrated by the studies presented above in transfected Cos-7 cells, it is clear that the presence of the "L" insert confers the protein the ability to concentrate in the cell nucleus, while the absence of the insert renders this protein mainly cytoplasmic.

While this work was in progress, Fu et al. [2001] reported a similar study using the four chicken splice variants of CK1α transfected into Cos-7 and 3T3 cells. These authors also found that the presence of the "L" insert targets the enzyme to the nucleus. In contrast, the protein lacking the "L" insert was excluded from the

nucleus in this work. This latter observation is at variance with our results, in which CK1α is found to be predominantly cytoplasmic but is clearly not excluded from the nucleus and CK1αL shows a faint but consistent presence throughout the cytoplasm. A possible reason for this discrepancy is the type of tag attached to the CK1α constructs. Fu et al. [2001] used a fusion with the green fluorescent protein while we used the small [HA] epitope. The molecular masses of CK1α and CK1αL are 37.5 and 40.8 kDa, respectively, which could allow passive diffusion of the proteins into the nucleus [Allen et al., 2000]. The addition of a large fusion protein such as GFP may prevent such passive transport into this organelle. The HA epitope adds only 1 kDa of mass, which would not interfere with this transport.

The "L" insert was also found to contain a sequence, C L E S P V G K R, which is similar to a sequence found in rhodopsin kinase C L E Q P I G K R. This similarity is intriguing because CK1α, like rhodopsin kinase, has been found to phosphorylate the rhodopsin receptor and other "G" protein-coupled receptors in a ligand-dependent manner [Tobin et al., 1997; Waugh et al., 1999]. The authors of these reports have not specified which variant of CK1α is involved in those phosphorylations.

Another rather important difference between the variants was observed on the turnover of the proteins in experiments in which transfected cells were labeled with [^{35}S], followed by a chase with cold medium. The decay rate of the labeled CK1αL protein was four times that of CK1α. This is an important factor in governing the relative abundance of the splicing variants in cells that express these various forms of CK1α.

In addition to the interesting effects on cellular localization and degradation rate, the "L" insertion exerts some other effects on the biochemical properties of the protein. The results presented above demonstrate that the nucleotide binding pocket of the enzyme is affected by the "L" insert, increasing both the apparent Km for ATP and the I_{50} concentration of the CKI-7 inhibitor, which competes for the nucleotide-binding site. This insert also decreases the thermal stability of the enzyme, rendering it four times more thermolabile than the variants that lack this sequence. Zhang et al. [1996] had previously reported that CK1αL displayed a much higher Km for phosvitin than CK1α, while Yong et al. [2000] reported the inverse situation. We observed no significant difference in the apparent Km for β-casein, phosvitin, and the model peptide specific for CK1 phosphorylation among the different splice variants. The "L" insert-containing variants did, however, exhibit a higher specific activity towards β-casein, in concordance with the results of Zhang et al. [1996]. It is possible that there may exist differences in the relative activity of the CK1α variants with other more physiological substrates that we have not tested. One possibility for this difference may be that the "L" or "S" inserts might provide "docking sites" for some substrates [Holland and Cooper, 1999]. However, no great differences in the activity of the variants were observed when tested with NF-AT4 peptides that contain a functional "docking site" that stimulates phosphorylation in a separate non-canonical site [Marin et al., 2002].

Most of the work that has implicated CK1α in important physiological processes such as NF-AT4 phosphorylation [Zhu et al., 1998], Wnt signaling, and the phosphorylation of β-catenin [Liu et al., 2002] and α-centaurin binding [Dubois et al., 2001] has not concerned itself with specifying the splice variant of CK1α responsible for the activity observed. The work of Fu et al. [1999], however, has shown that only the short variants, CK1α and CK1αS, bind to neurofilaments obtained from chicken neurons. It seems pertinent, therefore, to further define the functional difference of these protein products of the same gene.

REFERENCES

Allen TD, Cronshaw JM, Bagley E, Kiseleve E, Goldberg MW. 2000. The nuclear pore complex: Mediator of translocation between nucleus and cytoplasm. J Cell Sci 113:1651–1659.

Allende CC, Allende JE. 1995. Protein kinase CK2: An enzyme with multiple substrates and a puzzling regulation. FASEB J 9:313–323.

Cegielska A, Fish K, Rivers A, Virshup DM. 1998. Autoinhibition of casein kinase I ε (CKIε) is relieved by protein phosphatases and limited proteolysis. J Biol Chem 273: 1357–1364.

Chijiwa T, Hagiwara M, Hidaka H. 1989. A newly synthesized selective casein kinase I inhibitor, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and affinity purification of casein kinase I from bovine testis. J Biol Chem 264:4924–4927.

Dubois T, Kerai P, Zemlickova E, Howell S, Jackson TR, Venkateswarlu K, Cullen PJ, Theibert AB, Larose L, Roach PJ, Aitken A. 2001. Casein kinase I associates with members of the centaurin-alpha family of phosphatidylinositol 3,4,5-trisphosphate-binding proteins. J Biol Chem 276:18757–18764.

Fish KJ, Cegielska A, Getman M, Landes GM, Virshup DM. 1995. Isolation and characterization of human casein kinase Iε (CKI), a novel member of the CKI gene family. J Biol Chem 270:14875–14883.

Flotow H, Roach PJ. 1989. Synergistic phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase and casein kinase I. Implications for hormonal regulation of glycogen synthase. J Biol Chem 264:9126–9128.

Flotow H, Roach PJ. 1991. Role of acidic residues as substrate determinants for casein kinase I. J Biol Chem 266: 3724–3727.

Flotow H, Graves PR, Wang A, Fiol CJ, Roeske RW, Roach PJ. 1990. Phosphate groups as substrate determinants for casein kinase I action. J Biol Chem 265:14264–14269.

Frame S, Cohen P. 2001. GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359:1–16.

Fu Z, Green CL, Bennett GS. 1999. Relationship between casein kinase I isoforms and a neurofilament-associated kinase. J Neurochem 18:745–757.

Fu Z, Chakraborti T, Morse S, Bennett GS, Shaw G. 2001. Four casein kinase I isoforms are differentially partitioned between nucleus and cytoplasm. Exp Cell Res 269: 275–286.

Graves PR, Roach PJ. 1995. Role of COOH-terminal phosphorylation in the regulation of casein kinase I δ. J Biol Chem 270:21689–21694.

Graves PR, Haas DW, Hagedorn CH, DePaoli-Roach AA, Roach PJ. 1993. Molecular cloning, expression and characterization of a 49-kilodalton casein kinase I isoform from rat testis. J Biol Chem 268:6394–6401.

Green CL, Bennet GS. 1998. Identification of four alternatively spliced isoforms of chicken casein kinase I alpha that are all expressed in diverse cell types. Gene 216: 189–195.

Gross SD, Anderson RA. 1998. Casein kinase I: Spatial organization and positioning of a multifunctional protein kinase family. Cell Signal 10:699–711.

Hanks SK, Hunter T. 1995. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. FASEB J 9:576–596.

Harwood AJ. 2001. Regulation of GSK-3: A cellular multiprocessor. Cell 105:821–824.

Ho U, Mason S, Kobayashi R, Hoekstra M, Andrews B. 1997. Role of the casein kinase I isoform, Hrr25, and the cell cycle regulatory transcription factor, SBF, in the transcriptional response to DNA damage in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 94:581–586.

Holland PM, Cooper JA. 1999. Protein modification: Docking sites for kinases. Curr Biol 9:329–331.

Kloss B, Rothenfluh A, Young MW, Saez L. 2001. Phosphorylation of period is influenced by cycling physical association of double-time, period and timeless in the *Drosophila* clock. Neuron 30:699–706.

Liu F, Ma X-H, Ule J, Bibb JA, Nishi A, DeMaggio A, Yan Z, Nairn AC, Greengard P. 2001. Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. Proc Natl Acad Sci USA 98:11062–11068.

Liu C, Li Y, Semenov M, Han C, Baeg G-H, Tan Y, Zhang Z, Lin X, He X. 2002. Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108:837–847.

Marin O, Burzio V, Boschetti M, Meggio F, Allende CC, Allende JE, Pinna LA. 2002. Structural features underlying the multisite phosphorylation of the A domain of the NF-AT4 transcription factor by protein kinase CK1. Biochemistry 41:618–627.

Meggio F, Perich JW, Reynolds EC, Pinna LA. 1991. A synthetic β-casein phosphopeptide and analogues as model substrates for casein kinase-1, a ubiquitous, phosphate directed protein kinase. FEBS Lett 283:303–306.

Meggio F, Perich JW, Marin O, Pinna LA. 1992. The comparative efficiencies of the Ser(*P*)- and Tyr(*P*)-residues as specificity determinants for casein kinase-1. Biochem Biophys Res Commun 182:1460–1465.

Murakami A, Kimura K, Nakano A. 1999. The inactive forms of a yeast casein kinase I suppresses the secretory defect of the sec12 mutant. Implication of negative regulation by the Hrr25 kinase in the vesicle budding from the endoplasmic reticulum. J Biol Chem 274:3804–3810.

Peters JM, McKay RM, McKay JP, Graff JM. 1999. Casein kinase I transduces Wnt signals. Nature 401:345–350.

Pulgar V, Tapia C, Vignolo P, Santos J, Sunkel C, Allende CC, Allende JE. 1996. The recombinant α isoform of protein kinase CK1 from *Xenopus laevis* can phosphorylate tyrosine in synthetic peptides. Eur J Biochem 260: 520–526.

Pulgar V, Marin O, Meggio F, Allende CC, Allende JE, Pinna LA. 1999. Optimal sequences for non-phosphate-directed phosphorylation by protein kinase CK1 (casein kinase-1)—a re-evaluation. Eur J Biochem 260:520–526.

Rowles J, Slaughter C, Moomaw C, Hsu J, Cobb MH. 1991. Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. Proc Natl Acad USA 88:9548–9552.

Sanger F, Nicklen S, Coulson AR. 1997. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Tapia C, Featherstone T, Gomez C, Taillon-Miller P, Allende CC, Allende JE. 1994. Cloning and chromosomal localization of the gene coding for human protein kinase CK1. FEBS Lett 349:307–312.

Tellez R, Gatica M, Allende CC, Allende JE. 1990. Copolymers of glutamic acid and tyrosine are potent inhibitors of oocyte casein kinase II. FEBS Lett 265:113–116.

Tobin AB, Totty NF, Sterlin AE, Nahorsky SR. 1997. Stimulus-dependent phosphorylation of G protein-coupled receptors by casein kinase 1α. J Biol Chem 272:20844–20849.

Waugh MG, Challiss J, Berstein G, Nahorski SR, Tobin AB. 1999. Agonist-induced desensibilization and phosphorylation of m1-muscarinic receptors. Biochem J 338:175–183.

Westerfield M. 1993. The zebrafish book; a guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon Press. Eugene, Oregon.

Yong TJ, Gan YY, Toh B-H, Sentry JW. 2000. Human CK1αL and CK1αS are econded by both 2.4 and 4.2 kb transcripts, the longer containing multiple RNA-destabilising elements. Biochem Biophys Acta 1492:425–433.

Zhang J, Gross SD, Schroeder MD, Anderson RA. 1996. Casein kinase I α and αL: Alternative splicing-generated kinases exhibit different catalytic properties. Biochemistry 35:16319–16327.

Zhu J, Shibasaki F, Price R, Guillemot J-C, Yano T, Dötsch V, Wagner G, Ferrara P, McKeon F. 1998. Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. Cell 93:851–861.