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Expression and gene knockdown of zebrafish Ca^{2+}/calmodulin-dependent protein kinase Iδ-LL

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase Iδ (CaMKIδ) is expressed ubiquitously, but little is known about its physiological functions. Recently, we cloned and characterized two splice variants of zebrafish (*Danio rerio*) CaMKIδ (CaMKIδ-S/L). In the present study we cloned a new CaMKIδ isoform, CaMKIδ-LL, encoded by a different gene from CaMKIδ-S/L. While the catalytic domain of CaMKIδ-LL showed 86% identity that of CaMKIδ-S/L, it had a unique C-terminal sequence. To clarify the functional role of CaMKIδ-LL, we investigated the biological significance of this new isoform during zebrafish embryogenesis. Although CaMKIδ-LL exhibited essentially the same catalytic properties and substrate specificities as the other CaMKIδ isoforms, it showed different temporal and spatial expression. During zebrafish embryogenesis, RT-PCR analysis detected CaMKIδ-LL expression after 48 h post-fertilization. Western blotting in adult zebrafish demonstrated that CaMKIδ-LL is expressed in the brain, the eye, and, abundantly, in fins. Knockdown of CaMKIδ-LL expression using morpholino-based antisense oligonucleotides resulted in an increase in abnormal embryos with small fins and underdeveloped cartilage. These phenotypes were rescued by co-injection with recombinant CaMKIδ-LL. These results clearly indicated that CaMKIδ-LL plays an important role in the generation of cartilage and fins during zebrafish embryogenesis.

Introduction

Protein kinases play pivotal roles in various signaling pathways and participate in diverse cellular processes [1]. The increase of Ca^{2+} concentration in response to extracellular stimuli activates various Ca^{2+}/calmodulin-dependent (CaM-dependent)[1] enzymes involved in cellular signaling, such as Ca^{2+}/CaM-dependent protein kinases (CaMKs). A variety of cellular functions are regulated by Ca^{2+} signaling through CaMK cascades, which consist of multifunctional CaMKs: CaMKI, CaMKII, and CaMKIV. CaMKII is regulated by Ca^{2+}/CaM-dependent autophosphorylation [2–5], while CaMKI and CaMKIV are activated by phosphorylation of Thr residues in the activation loops by an upstream kinase, CaMK kinase (CaMKK) [6–9].

In our previous study, we developed a unique expression screening technique for protein kinases with the aid of kinase-specific monoclonal antibodies [10,11]. Using this technique, we found two splice variants of CaMKIδ with different C-terminal sequences that were present in zebrafish embryos at 72 h post-fertilization (hpf) [12]. The open reading frame of the shorter transcript comprised 1176 nucleotides and encoded a protein of 392 amino acid residues, designated as CaMKIδ-L. On the other hand, the longer transcript had an insertion of 29 bases between the 1077th and 1078th bases, and the position of the stop codon was changed by a frame shift. As a result, this gene encodes a protein of 368 amino acid residues and was designated as CaMKIδ-S. The N-terminal sequence (1–359 amino acids) of CaMKIδ-S/L was identical. These isoforms are expressed at different stages in early embryogenesis and showed different tissue distribution in adult fish. Gene knockdown of CaMKIδ-S/L by morpholino-based antisense oligonucleotide (AS-MO) induced significant morphological abnormalities in zebrafish embryos, producing small heads and short bodies, and this phenotype could be rescued by co-injection with recombinant CaMKIδ-S/L. These results indicate that these CaMKIδ isoforms play crucial roles in zebrafish embryogenesis.

In the present study, we identified a new CaMKIδ isoform, CaMKIδ-LL, encoded by a different gene from CaMKIδ-S/L. Although the amino acid sequence of the catalytic domain of CaMKIδ-LL showed 86% identity with CaMKIδ-S/L, CaMKIδ-LL has a unique C-terminal sequence. Therefore, we expressed CaMKIδ-LL in *Escherichia coli* and investigated its biochemical properties. CaMKIδ-LL began to appear at 48 hpf during early embryogenesis and was expressed in the brain, eye and fins of the adult fish. Knockdown of the

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[1] *Abbreviations used:* CaM, calmodulin; CaMKI, Ca^{2+}/calmodulin-dependent protein kinase I; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; DIG, digoxigenin; hpf, hours post-fertilization; MBP, myelin basic protein; MO, morpholino oligonucleotide.

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CaMKIδ-LL gene expression by AS-MO induced significant morphological abnormalities in zebrafish embryos, such as small fins and underdeveloped cartilage. This phenotype could be rescued by co-injection with recombinant CaMKIδ-LL protein. These results indicate that CaMKIδ-LL has important functions in the process of cartilage and fin formation during early embryogenesis in zebrafish.

Materials and methods

Materials

ATP, Cy3-labeled anti-mouse IgG, Cy5-labeled anti-rabbit IgG, a monoclonal anti-β-tubulin antibody, bovine serum albumin and myelin basic protein (MBP) from bovine brain were purchased from Sigma Chemicals. [γ-^{32}P]ATP (1 1 1 TBq/mmol) and glutathione Sepharose 4B were purchased from PerkinElmer and GE Healthcare Bio-Sciences, respectively. Goat anti-mouse IgG and goat anti-rabbit IgG, conjugated with horseradish peroxidase, were obtained from ICN Pharmaceuticals. An anti-His_6 antibody was purchased from Wako. An anti-phosphothreonine antibody (42H4) was obtained from Cell Signaling Technology. Recombinant proteins (rat CaM [13], mouse CaMKKα [14], zebrafish CaMKIδ-S/L [12], rat CaMKIα [15] and λPPase [16]) were expressed in *E. coli* and purified as described previously.

Cloning the CaMKIδ-LL cDNA by rapid amplification of cDNA ends (RACE)

A SMART RACE cDNA Amplification Kit (Clontech) was used to obtain a full-length coding sequence for CaMKIδ-LL using primers based on a GenBank zebrafish EST database clone (Accession No.: BC160632). The 5′-RACE first-strand cDNA was synthesized from mRNA isolated from the zebrafish brain with Superscript II reverse transcriptase using a SMARTer™ RACE cDNA Amplification Kit (Clontech). A sense primer (5′-GTA ACG GTA CAA CCG GGG AAG GCG A-3′) and an antisense primer (5′-GCA GGA GTG CAT TAG GCG TGC GC-3′) were designed from the sequences flanking the open reading frame. The full-length cDNA was prepared by PCR using these primers and 5′-RACE ready cDNA library as a template with Pyrobest DNA polymerase (TaKaRa Bio). The PCR product was cloned into pGEM-T Easy vector (Promega) and designated as pGEMCaMKIδ-LL.

Construction of plasmids

To generate the plasmid pETCaMKIδ-LL, the following primers were used for PCR with pGEMCaMKIδ-LL as a template: sense primer (5′-AAA GCT AGC ATG GCT CGG GAG AAC GGA GA-3′) and antisense primer (5′-TTT CTC GAG CTT GGT CCC AGT TAC CAC TGT GC-3′). The *Nhe*I (underlined)-*Xho*I (double-underlined) fragment was inserted into the *Nhe*I-*Xho*I sites of pET-23a(+) (Novagen). The plasmids pETCaMKIδ-S and pETCaMKIδ-L were prepared as described previously [12].

Mutagenesis of Lys-54 was performed by inverse PCR [17] with sense primer (5′-GCA TGC ATC CCT AAA AAA GCT CTG AAG-3′; underlined bases show the site of the mutation) and antisense primer (5′-CAC AGC GTA CAT CTT TCC CGT GG-3′) and pETCaMKIδ-LL as a template. The 5′-ends of the PCR fragments were then phosphorylated by T4 polynucleotide kinase (Nippon Gene) and self-ligated by T4 DNA ligase (Nippon Gene). The recombinant plasmid obtained was designated as pETCaMKIδ-LL(K54A).

For mammalian cells, the following plasmids were prepared: pcCaMKIδ-LL was generated by PCR using primers (5′-AAA AAG CTT GTT ATG GCT CGG GAG AAC GGA GA-3′ and 5′-TTT CTC GAG CGC TTG GTC CCA GTT ACC ACT GTG C-3′) and pETCaMKIδ-LL as a template. The *Hin*dIII (underlined)-*Xho*I (double underlined) fragment was inserted into *Hin*dIII-*Xho*I sites of pcDNA3.1(+)/*myc*-His B (Invitrogen). The plasmids pcCaMKIδ-S and pcCaMKIδ-L were prepared as described previously [12].

Expression and purification of recombinant CaMKIδ

The plasmid pETCaMKIδ-LL was introduced into *E. coli* strain BL21 (DE3). The transformed bacteria were grown at 37 °C to an A_{600} of 0.6–0.8, and then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 24 h at 25 °C, the bacteria were harvested by centrifugation and suspended in buffer A [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40, and 1 mM PMSF]. After sonication, cell debris was removed by centrifugation (20,000*g*) at 4 °C for 10 min, and the supernatant was loaded on a HiTrap Chelating HP column (GE Healthcare Bio-Sciences) pre-equilibrated with buffer A. The column was washed sequentially with buffer A, buffer A containing 20 mM imidazole, buffer A containing 50 mM imidazole, and then eluted with buffer A containing 200 mM imidazole. The latter fractions were pooled, dialyzed against 20 mM Tris–HCl (pH 7.5) containing 0.05% Tween 40 and 1 mM 2-mercaptoethanol, and used for characterization of the enzyme.

Production of antibodies

Antibodies against phospho-CaMKI at Thr-177 were produced by immunizing BALB/c mice with an antigenic phosphopeptide (for anti-phospho-CaMKI) [18]. Isoform-specific antibodies against CaMKIδ-S and CaMKIδ-L were prepared as described previously [12]. To produce antibodies against CaMKIδ-LL, GST-fused CaMKIδ-LL(334–433) was expressed in *E. coli* and CaMKIδ-LL (334–433) was purified by glutathione Sepharose 4B and PreScission protease, as described previously [9]. A specific antibody against CaMKIδ-LL was raised by immunizing a rabbit with purified CaMKIδ-LL(334–433) as an antigen, according to the procedure described previously [19]. The anti-CaMKIδ-LL antibody was purified by affinity chromatography using CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences) coupled by CaMKIδ-LL(334–433). Affinity purification of the CaMKIδ-S and CaMKIδ-L antibodies was performed using the SulfoLink Immobilization Kit for Peptides (Thermo Scientific).

Fish maintenance

Zebrafish were maintained at 26 °C and embryos were collected from natural crosses of wild-type fish. Embryos were maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) at 28 °C. Embryos were staged according to hpf at 28 °C and by morphological criteria [20].

Preparation of crude extracts from zebrafish embryos and adult tissues

Crude extracts from embryos at each sampling point were prepared as follows: dechorionated embryos ($N = 40$) were suspended in 200 μl of ice cold homogenizing buffer containing 5 mM Tris–HCl buffer (pH 7.5), 0.5 mM EGTA, 1 mM EDTA,1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml antipain, and homogenized by sonication (Tomy, Handy Sonic UR-20P). Crude extracts from adult tissues were prepared as above except that five volumes of homogenizing buffer were used and a Polytron homogenizer was used (Kinematica). The homogenates were centrifuged at 20,000*g* at 4 °C for 10 min, and the supernatants were used as crude extracts.