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A minimum size homologue of Ca^{2+}/calmodulin-dependent protein kinase IV naturally occurring in zebrafish

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Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV is a multifunctional Ser/Thr protein kinase that is predominantly expressed in the nuclei of neurons. CaMKIV consists of a catalytic domain and a regulatory (Ca^{2+}/calmodulin binding and autoinhibitory) domain, which are located in the N-terminal and central regions, respectively. Here, we identified the zebrafish homologue of CaMKIV (zCaMKIV) on the basis of biochemical characterization. zCaMKIV showed similar biochemical properties as well as tissue and subcellular distributions to rat CaMKIV (rCaMKIV). However, zCaMKIV had a fairly small size with a molecular mass of about 40 kDa, and was devoid of a region corresponding to the C-terminal domain of rCaMKIV. Since zCaMKIV is composed of regions that are nearly equivalent to only a catalytic and a regulatory domain, it should represent a minimum size homologue possessing CaMKIV function. zCaMKIV and rCaMKIV differed in their substrate specificities, since rCaMKIV preferred histone H1 over myelin basic protein, while zCaMKIV did not. Moreover, zCaMKIV was more readily dephosphorylated by zebrafish nuclear CaMK phosphatase (CaMKP-N) than rCaMKIV. These results suggest that the C-terminal region of CaMKIV plays a role in interacting with its target and modulator proteins.

Keywords: CaM kinase/CaMKIV/CaMKP-N/substrate specificity/zebrafish.

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CaMKP-N, nuclear Ca^{2+}/calmodulin-dependent protein kinase phosphatase; CREB, cyclic AMP-responsive element-binding protein; DIG, digoxigenin; MBP, myelin basic protein; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.05% Tween 20.

The intracellular Ca^{2+} receptor calmodulin (CaM) and its downstream Ca^{2+}/CaM-dependent protein kinases (CaMKs) I, II and IV connect transient increases in intracellular Ca^{2+} with physiological processes such as proliferation, development and differentiation (*1–3*). CaMKIV is a multifunctional Ser/Thr protein kinase that is predominantly expressed in the nuclei of neurons in certain brain regions, including the cortex, cerebellum and hippocampus (*4–7*). This predominantly nuclear protein kinase regulates transcription factors including cyclic adenosine monophosphate-responsive element-binding protein (CREB) (*8*), CREB-binding protein (*9*, *10*) and other proteins (*11*, *12*). A study of transgenic mice carrying dominant-negative CaMKIV alleles conferring defects in CREB phosphorylation revealed that these mice exhibit a disruption of late-phase long-term potentiation and are impaired in the consolidation/retention phase of hippocampus-dependent memory (*13*). Analyses of mice deficient in CaMKIV revealed that the CaMKIV-mediated pathway plays important roles in the function and development of both the cerebellum and hippocampal CA1 neurons (*14*, *15*).

The regulation of CaMKIV has been a subject of considerable interest. Similar to other CaMKs, the active site of CaMKIV is sterically blocked by an autoinhibitory domain that prevents substrate binding to the enzyme. Binding of Ca^{2+}/CaM to this region relieves the autoinhibition by dissociating the autoinhibitory domain from the catalytic domain, and exposes a critical Thr residue in the activation loop, leading to its phosphorylation by CaMK kinase (CaMKK) (*16*, *17*). This phosphorylation markedly increases CaMKIV activity and leads to the generation of Ca^{2+}/CaM-independent (autonomous) activity. Autophosphorylation of Ser residues in the N-terminal region also relieves the intramolecular steric inhibition (*18*). However, the roles of the C-terminal region of CaMKIV are not well defined.

In a previous study, we showed that antisense knockdown of nuclear CaMK phosphatase (CaMKP-N) induced significant morphological abnormalities in zebrafish embryos (*19*). We assumed that this abnormal phenotype was caused by aberrant activation of CaMKIV, because CaMKP-N is considered to be one of the downregulators of CaMKIV activity. To clarify the molecular mechanisms underlying the abnormal phenotype of these embryos, it is a prerequisite to know the molecular properties of the zebrafish homologue of CaMKIV (zCaMKIV). However, biochemical characterization of zCaMKIV has not been reported.

In the present study, we carried out molecular cloning and expression of zCaMKIV to characterize its biochemical properties. We found that zCaMKIV existed in zebrafish as a truncated form that is devoid of a region corresponding to the C-terminal domain of rat CaMKIV (rCaMKIV). We analysed the enzymatic properties of zCaMKIV, and compared them with those of rCaMKIV. The activation mechanism, tissue distribution and subcellular localization of zCaMKIV were similar to those of rCaMKIV. However, the substrate specificity of zCaMKIV was somewhat different from that of rCaMKIV. Furthermore, phosphorylated zCaMKIV served as a better substrate for CaMKP-N than phosphorylated rCaMKIV. Taken together, the present findings suggest that the C-terminal region of rCaMKIV, which is absent from zCaMKIV, is involved in the regulation of both its substrate specificity and its susceptibility to dephosphorylation by CaMKP-N.

Materials and Methods

Materials

Adenosine triphosphate (ATP), bovine serum albumin, myelin basic protein (MBP) from bovine brain, poly-L-lysine, Cy3-conjugated anti-mouse IgG, anti-CREB and anti-phospho(Ser-133)-CREB antibodies were purchased from Sigma. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were obtained from Pierce. [γ-^{32}P]ATP (5000 Ci/mmol) was purchased from GE Healthcare Bio-Sciences. Syntide-2 was synthesized using a Shimadzu PSSM-8 automated peptide synthesizer, and purified by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column (ODS-80 Tm; Tosoh). Histone H1 from calf thymus was obtained from Calbiochem. Recombinant rat CaM (*20*), mouse CaMKK (*21*), rat CREB (*22*) and zebrafish CaMKP-N (*19*) were expressed in *Escherichia coli* and purified as described previously. Anti-zCaMKIV and anti-phospho(Thr-183)-zCaMKIV monoclonal antibodies were generated using an antigenic phosphopeptide (LSKIVDDQVTMKTpVCGTPGYC; Peptide Institute Inc.) corresponding to amino acids 171–191 of zCaMKIV, essentially as described previously (*23*). Hybridoma cultures producing antibodies against non-phosphorylated zCaMKIV or phospho(Thr183)-zCaMKIV were selected by dot-immunobinding assays (*24*) using the non-phosphorylated peptide or phosphopeptide conjugated to poly-L-lysine as an antigen, respectively.

cDNA cloning of zCaMKIV

A cDNA for zCaMKIV (Accession No. NM_001017607) was identified by a homology search of the NCBI database using the amino acid sequence of rCaMKIVα(Accession No. NP_036859). A sense primer (5′-ACG GCC ACT CAT CAC CAA CA-3′) and an anti-sense primer (5′-CCT CTA TGA GGC AAC GCG TT-3′) were designed for sequences outside of the open reading frame. A full-length cDNA was prepared by polymerase chain reaction (PCR) using these primers and a zebrafish brain 5′-RACE ready cDNA library as a template with Pyrobest DNA polymerase (TaKaRa). The PCR product was cloned into a pGEM-T Easy vector (Promega), and five independent clones (pGEMzCaMKIV-1, -2, -3, -4 and -5) were sequenced.

Construction of plasmids

For zCaMKIV-His$_6$, the following primers were used for PCR with pGEMzCaMKIV-5 as a template: 5′-upstream primer (5′-GCT AGC ATG CTG AAA GTG ACA ATG CCC G-3′) and 3′-downstream primer (5′-CTC GAG AGA GCC CTC ATT CTG AAT CTG AGG-3′). The *Nhe*I (underlined)-*Xho*I (double-underlined) fragment was inserted into the *Nhe*I–*Xho*I sites of pET-23a(+) (Novagen) to generate the plasmid pET-zCaMKIV. For GST-zCaMKV-His$_6$, the following primers were used for PCR with pET-zCaMKIV as a template: 5′-upstream primer (5′-GAA TTC ATG CTG AAA GTG ACA ATG CCC G-3′) and 3′-downstream primer (LHis6/SalI, 5′-TTT GTC GAC TCA GTG GTG GTG GTG GTG GTG GTG-3′). The *Eco*RI (underlined)–*Sal*I (double-underlined) fragment was inserted into the *Eco*RI–*Sal*I sites of pGEX 6P-1 (GE Healthcare Bio-Sciences) to generate the plasmid pGEX-zCaMKIV. To express zCaMKIV in mammalian cells, pcDNA-zCaMKIV was prepared as follows. The cDNA fragment encoding C-terminal myc/His$_6$-tagged full-length zCaMKIV was amplified with PCR using specific primers (5′-AAG CTT GCC ATG CTG AAA GTG ACA ATG CC-3′ and 5′-CTC GAG GCA GAG CCC TCA TTC TGA ATC TG-3′) and pET-zCaMKIV as a template. The *Hin*dIII (underlined)–*Xho*I (double-underlined) fragment was inserted into the *Hin*dIII–*Xho*I sites of pcDNA3.1(+)-*myc*-His B (Invitrogen).

For GST-rCaMKIV(WT)-His$_6$, the following primers were used for PCR with pET-rCaMKIV (*19*) as a template: 5′-upstream primer (5′-GTC GAC TGA TGC TCA AAG TCA CGG TGC C-3′) and 3′-downstream primer (LHis6/SalI). The *Sal*I (underlined)-*Sal*I fragment was inserted into the *Sal*I site of pGEX 6P-1 to generate the plasmid pGEX-rCaMKIV(WT). For GST-rCaMKIV(1-344)-His$_6$, the following primers were used for inverse PCR (*25*) with pGEX-rCaMKIV(WT) as a template: 5′-upstream primer (5′-CAC CAC CAC CAC CAC CAC TGA-3′) and 3′-downstream primer (5′-GTT GGT GTG ACT GCT GCT GG-3′). The 5′-ends of the PCR fragment were phosphorylated by T4 polynucleotide kinase and self-ligated by T4 DNA ligase, and the resulting recombinant plasmid was designated pGEX-rCaMKIV(1-344).

For mammalian cells, pcDNA-rCaMKIα was prepared by PCR with specific primers (5′-GGT ACC GCC ATG CCA GGG GCA GTG GAA GGC-3′ and 5′-CCG CGG GTC CAT GGC CCT AGA GCT TGG GG-3′) using a rat brain 5′-RACE ready library as a template. The *Kpn*I (underlined)-*Sac*II (double-underlined) fragment was inserted into the *Kpn*I-*Sac*II sites of pcDNA3.1(+)-*myc*-HisB to generate pcDNA-rCaMKIα.

Expression of recombinant CaMKIVs in E. coli and purification

Expression vectors for recombinant CaMKIV proteins were introduced into *E. coli* strain BL21(DE3) (Novagen). The transformed bacteria were grown at 25°C for 24 h in growth medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside. The bacteria were harvested by centrifugation, and the recombinant proteins were purified essentially as described previously (*26*).

In vitro *activation of zCaMKIV by CaMKK*

Phosphorylation of zCaMKIV by CaMKK was carried out at 30°C for 30 min in a reaction mixture (10 µl) consisting of 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 µM CaM, 100 µg/ml zCaMKIV, 10 µg/ml CaMKK and 100 µM ATP. The reaction was initiated by adding ATP and terminated by 10-fold dilution with ice-cold 50 mM HEPES–NaOH (pH 7.5), 2 mg/ml bovine serum albumin, 10% ethylene glycol and 2 mM EDTA. The diluted sample was subjected to a protein kinase assay or western blotting analysis.

In vitro *kinase assay of zCaMKIV*

For kinase assays with MBP as a substrate, the kinase reaction was carried out at 30°C for 30 min in a solution (10 µl) containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 µM CaM, 100 µM [γ-^{32}P]ATP (2500–4200 cpm/pmol), 3 µg/ml zCaMKIV and 100 µg/ml MBP in the presence of 1 mM EGTA (for autonomous activity) or 1 mM $CaCl_2$. The reaction was initiated by adding the enzyme, and terminated by adding 10 µl of 2× SDS-PAGE sample buffer. After SDS-PAGE, the phosphorylated proteins were detected by autoradiography.

Phosphorylation of syntide-2 was carried out at 30°C for 10 min in a solution (20 µl) containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 µM CaM, 50 µM [γ-^{32}P]ATP, 1 µg/ml zCaMKIV and 40 µM syntide-2 in the presence of 1 mM EGTA (for autonomous activity) or 1 mM $CaCl_2$. The reaction was initiated by adding the enzyme, and terminated by spotting aliquots (15 µl) onto phosphocellulose paper (Whatman P-81), followed by several washes with 75 mM phosphoric acid. Phosphate incorporation into syntide-2 was determined by liquid scintillation counting of the filters.

Dephosphorylation of CaMKIV by CaMKP-N

GST-rCaMKIV (40 μg/ml) or GST-zCaMKIV (40 μg/ml) was phosphorylated in a solution (100 μl) containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM, 100 μM ATP and 8 μg/ml CaMKK. After termination of the reaction by the addition of 10 μl of 1 mg/ml bovine serum albumin and 10 μl of 20 mM EGTA, the phosphoproteins were desalted on a Sephadex G-50 spin column as described previously (*27*). Protein phosphatase assays were carried out for 10 min at 30°C in a solution (20 μl) containing 50 mM Tris–HCl (pH 8.0), 1 mM DTT, 5 mM $MnCl_2$, zCaMKP-N (0.5, 1, 2 or 10 μg/ml) and phospho-GST-rCaMKIV (5 μg/ml) or phospho- GST-zCaMKIV (5 μg/ml). The reaction was started by the addition of zCaMKP-N and terminated by the addition of 20 μl of 2× SDS-PAGE sample buffer. Each sample was then subjected to SDS-PAGE and analysed by western blotting using anti-phospho-CaMKIV antibody.

Phosphorylation of recombinant CREB by zCaMKIV

Phosphorylation of recombinant rat CREB (50 μg/ml) was carried out at 30°C for 30 min in a solution (30 μl) containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP in the presence or absence of 5 μg/ml zCaMKIV and 0.5 μg/ml CaMKK. The reaction was initiated by the addition of ATP and terminated by the addition of 30 μl of 2× SDS-PAGE sample buffer. The samples were then subjected to SDS-PAGE and analysed by western blotting using an anti-phospho-CREB antibody.

Phosphorylation of endogenous CREB on nitrocellulose membranes by zCaMKIV

A zebrafish brain extract was fractionated using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). The proteins (25 μg) in each fraction were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Protran BA85; Schleicher & Schuell). The membranes were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) for 1 h and incubated at 30°C for 3 h in a reaction mixture containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP with or without 10 μg/ml zCaMKIV and 1 μg/ml CaMKK. The membranes were then washed five times with PBST and probed with anti-CREB or anti-phospho-CREB antibodies.

Kinase assay on nitrocellulose membranes with a zebrafish brain extract as a substrate

Fractionated proteins (25 μg) from a zebrafish brain extract were subjected to SDS-PAGE and transferred to nitrocellulose membranes as described above. The membranes were washed with PBST for 1 h and then incubated at 30°C for 1 h in a reaction mixture containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP to avoid autophosphorylation of endogenous protein kinases. After three washes with PBST, the membranes were incubated at 30°C for 4 h in a reaction mixture containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM, 100 μM [γ-^{32}P]ATP and 5 μg/ml GST-CaMKIV activated by CaMKK. The membranes were extensively washed with PBST and phosphorylated proteins were detected by autoradiography.

Cell culture and transfection

Mouse neuroblastoma Neuro2a cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% heat-inactivated fetal calf serum at 37°C in a humidified incubator under a 5% CO_2/95% air atmosphere. Transfection of Neuro2a cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunocytochemistry of zCaMKIV

Transfected cells were cultured on cover glasses and treated with 3.7% formaldehyde in PBS for 20 min. After rinsing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum albumin in PBS. The samples were incubated with an anti-myc antibody (Invitrogen) diluted 1:1000 with 1% bovine serum albumin in PBS at 4°C overnight, followed by incubation with Cy3-conjugated anti-mouse IgG at room temperature for 2 h. Stained cells were observed with a confocal laser scanning microscope (TCS SP; Leica).

Preparation of a digoxigenin (DIG)-conjugated Multi-PK antibody

The Multi-PK antibody (M8C) (*28*) was purified from ascites fluid using protein A Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences). A DIG-conjugated Multi-PK antibody was prepared as follows. Purified Multi-PK antibody (250 μg) was diluted to 250 μl with PBS, followed by the addition of 10 μg of DIG-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche Diagnostics) in 10 μl of H_2O. The mixture was incubated at 4°C for 2 h and unreacted reagent was removed by dialysis against PBS with three changes of the buffer. The DIG-conjugated Multi-PK antibody solution was divided into aliquots and stored at −30°C until use.

Immunoprecipitation study

Extracts of zebrafish adult brain (1 mg of protein) and embryos (10 mg of protein) were dissolved in 50 mM Tris–HCl (pH 7.5) containing 1 mM phenylmethylsulphonyl fluoride and 10 μg/ml protease inhibitors (antipain, leupeptin, pepstatin and chymostatin). The supernatants were incubated with anti-zCaMKIV antibody-coupled protein G-Sepharose at 4°C for 2 h in an immunoprecipitation buffer consisting of 50 mM Tris–HCl (pH 7.5), 1% Triton X-100 and 0.5 M NaCl. After washing with immunoprecipitation buffer to remove unbound proteins, the Sepharose gel was incubated and boiled with 2× SDS-PAGE sample buffer, followed by centrifugation. The resulting supernatant was applied to SDS-PAGE, followed by western blotting analysis using the DIG-conjugated Multi-PK antibody (*28*).

Fish maintenance

Zebrafish, *Danio rerio*, were maintained at 26°C and embryos were collected from natural crosses of wild-type fish. The collected 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 26°C. The embryos were staged according to the hours post-fertilization (hpf) at 26°C and morphological criteria (*29*).

Other methods

SDS-PAGE was performed essentially according to the method of Laemmli (*30*) in slab gels consisting of a 10% or 15% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes and immunoreactive protein bands were detected essentially as described previously (*28*). CaM-overlay assays were carried out using DIG-labeled CaM as described previously (*21*). Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*31*). Nucleotide sequences were determined on both strands by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit Ver. 3.1 (Applied Biosystems) and a DNA Sequencer (Model 3100; Applied Biosystems).

Results

cDNA cloning of zebrafish CaMKIV

A cDNA encoding zCaMKIV was identified by a homology search of the NCBI database using the amino acid sequence of rCaMKIVα (Accession No. NP_036859) as a query. The full-length cDNA was obtained using a PCR-based strategy as described in MATERIALS AND METHODS section. The open reading frame of 1,092 bp encoded a polypeptide of 364 amino acids. A comparison of the predicted sequence of zCaMKIV protein with vertebrate CaMKIV protein sequences is shown in Fig. 1. zCaMKIV and the other CaMKIVs shared high degrees of homology (90–92%) within their catalytic and autoinhibitory/CaM-binding domains. The phosphorylation site for CaMKK in the

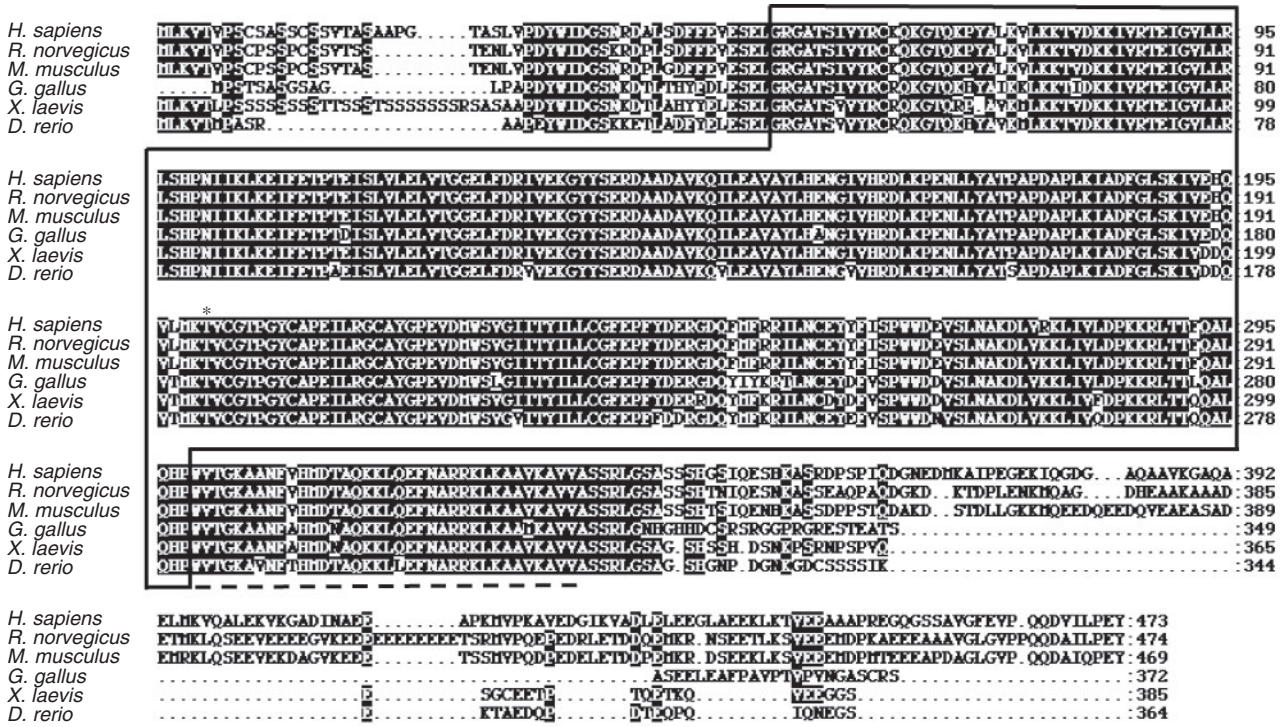

Fig. 1 Alignment of the zebrafish homologue of CaMKIV with other CaMKIVs. An amino acid sequence comparison of CaMKIVs (accession numbers: human (*Homo sapiens*), NP_001735; rat (*Rattus norvegicus*), NP_036859; mouse (*Mus musculus*), NP_033923; chicken (*Gallus gallus*), NP_001029985; frog (*Xenopus laevis*), NP_001079220; zebrafish (*Danio rerio*), NP_001017607) is shown. The respective amino acid numbers are shown on the right. Identical amino acid residues in the species are shaded in black. The catalytic domain is indicated by a box. The phosphorylation site in CaMKIV responsible for activation by CaMKK is indicated by an asterisk. The regulatory region containing the autoinhibitory domain and the calmodulin-binding domain is indicated by a broken line.

activation loop of CaMKIV (Thr-200 in human, Thr-196 in rat and mouse, Thr-185 in chicken and Thr-204 in frog) was conserved in zCaMKIV (Thr-183) (Fig. 1). However, zCaMKIV lacked the Ser residues corresponding to the autophosphorylation sites of human CaMKIV (Ser-12 and Ser-13) at its N-terminal region (*18*), as well as a region corresponding to the C-terminal region of rCaMKIV. Judging from the deduced amino acid sequences, the molecular mass and p*I* of zCaMKIV were calculated to be 40,619 Da and 7.16, respectively, indicating that the zebrafish homologue has a considerably lower molecular mass and a higher p*I* value than the rat homologue, of which the molecular mass and p*I* were calculated to be 53,148 Da and 4.70, respectively.

As shown in Fig. 1, vertebrate CaMKIVs can be classified into two types based on their C-terminal structures as follows: the longer CaMKIVs (human, rat and mouse) composed of ~470 amino acids and the shorter CaMKIVs (chicken, frog and zebrafish) composed of 360–380 amino acids lacking the C-terminal region of the former. Although the biochemical properties of the longer CaMKIVs, such as rCaMKIV, have been extensively studied, there is little detailed information available for the shorter CaMKIVs including zCaMKIV. Therefore, we decided to explore the biochemical properties of zCaMKIV to clarify whether it did indeed act as CaMKIV irrespective of its low molecular mass.

Activation mechanisms of zCaMKIV

It is widely accepted that mammalian CaMKIVs are activated by Ca^{2+}/CaM binding and phosphorylation of Thr in the activation loop by the upstream kinase CaMKK (*16*, *17*, *32*). We carried out CaM-overlay assays to examine the Ca^{2+}/CaM-binding activity of zCaMKIV. As shown in Fig. 2A, zCaMKIV was detected in the presence of $CaCl_2$, but not in the presence of excess EGTA. Similar results were obtained for rCaMKIV (Fig. 2A). Next, we investigated the phosphorylation of zCaMKIV by CaMKK by western blotting analysis with an anti-phospho(Thr-183)-zCaMKIV monoclonal antibody, which recognized the activation loop of zCaMKIV only when Thr-183 was phosphorylated. zCaMKIV was detected by the antibody when it was incubated under the phosphorylating conditions together with CaMKK, but not when it was incubated without CaMKK (Fig. 2B). To confirm that zCaMKIV phosphorylated by CaMKK was an active form of the enzyme, the kinase activities of phosphorylated and non-phosphorylated zCaMKIV were assayed using MBP as a substrate. Non-phosphorylated zCaMKIV did not phosphorylate MBP in the absence of Ca^{2+}/CaM, but weakly phosphorylated it in their presence. The Ca^{2+}/CaM-dependent phosphorylation of MBP was largely stimulated by CaMKK-catalysed phosphorylation of zCaMKIV. Moreover, MBP was phosphorylated even in the absence of Ca^{2+}/CaM when zCaMKIV had been preliminarily phosphorylated by

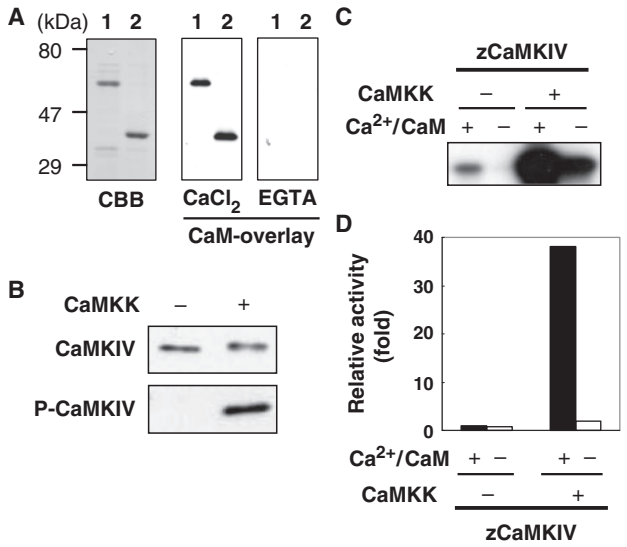

Fig. 2 Activation mechanisms of zCaMKIV. (A) Purified recombinant rCaMKIV-His_6 (lane 1) and zCaMKIV-His_6 (lane 2) were separated by 10% SDS-PAGE and then stained with Coomassie Brilliant Blue R-250 (CBB) or transferred to nitrocellulose membranes (Ca^{2+} or EGTA). The membranes were incubated with DIG-conjugated CaM and an anti-DIG antibody in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. (B) zCaMKIV (100 μg/ml) was incubated with or without CaMKK (10 μg/ml) for 30 min at 30°C in a solution containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP. The samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-CaMKIV or anti-phospho-CaMKIV antibodies. (C) The kinase activities of zCaMKIV incubated with or without CaMKK were measured using MBP as a substrate in the presence or absence of Ca^{2+}/CaM. Phosphorylation of MBP was detected by autoradiography. (D) The kinase activities of zCaMKIV incubated with or without CaMKK were measured using syntide-2 as a substrate in the presence or absence of Ca^{2+}/CaM. The radioactivity incorporated into syntide-2 was measured using a liquid scintillation counter.

CaMKK (autonomous activity) (Fig. 2C). When syntide-2 was used as a substrate, phosphorylated zCaMKIV showed about 40-fold higher activity than non-phosphorylated zCaMKIV in the presence of Ca^{2+}/CaM (Fig. 2D). These results indicate that zCaMKIV is strongly activated by both Ca^{2+}/CaM binding and CaMKK-catalysed phosphorylation of Thr in the activation loop, and that phosphorylated zCaMKIV has autonomous (Ca^{2+}/CaM-independent) activity. These characteristics are quite similar to those of mammalian CaMKIVs (*1–3*, *32*).

Subcellular localization of zCaMKIV

Mammalian CaMKIVs are mainly localized in the nuclei of neuronal cells (*33*). To investigate whether zCaMKIV is also localized in nuclei similar to mammalian CaMKIVs, rCaMKIV, zCaMKIV and rCaMKIα (which is known to be cytosolic) were transiently expressed in Neuro2a cells, and their subcellular localizations were examined by indirect immunofluorescence. rCaMKIα was exclusively localized in the cytosol (Fig. 3A), whereas zCaMKIV and rCaMKIV were mainly localized in the nuclei (Fig. 3B and C). Although more zCaMKIV appeared to be localized in the cytosol than rCaMKIV, a substantial amount of zCaMKIV existed in the nuclei as well as rCaMKIV.

Tissue distribution of zCaMKIV and its temporal expression during embryogenesis

To gain insights into the physiological roles of zCaMKIV, we investigated the tissue distribution of zCaMKIV by western blotting analysis with an anti-zCaMKIV monoclonal antibody. We found that zCaMKIV protein was only expressed in the brain, and had a molecular mass of about 40 kDa (Fig. 4A). These findings confirmed that the zCaMKIV cloned in this study naturally occurred in the zebrafish brain with the expected molecular mass. Next, we analysed the temporal expression of zCaMKIV during embryonic development. zCaMKIV was immunoprecipitated from lysates of embryos with an anti-CaMKIV antibody and detected by western blotting analysis. zCaMKIV was weakly expressed in the embryo at the 48 and 72 hpf stages, and strongly expressed in the embryo at the 96 hpf stage (Fig. 4B). These results suggest that zCaMKIV plays some roles in the embryogenesis in zebrafish.

zCaMKIV phosphorylates Ser-133 of CREB

CREB is believed to be one of the physiological substrates for CaMKIV, and the site in CREB phosphorylated by rCaMKIV is Ser-133 (*34*). To clarify whether zCaMKIV also phosphorylates Ser-133 of CREB, recombinant rat CREB was incubated with zCaMKIV in the presence or absence of CaMKK under the phosphorylating conditions. As shown in Fig. 5A, CREB was efficiently phosphorylated by zCaMKIV in the presence of CaMKK. To examine whether zCaMKIV also phosphorylates endogenous CREB in a zebrafish brain extract, the extract was fractionated according to a standard subcellular fractionation protocol and transferred to nitrocellulose membranes. The membranes were incubated with or without zCaMKIV and CaMKK under the phosphorylating conditions as described in MATERIALS AND METHODS section. When the membranes were probed with an anti-phospho-CREB antibody, prominent positive bands were detected only when the membranes were incubated with both zCaMKIV and CaMKK. The mobilities of the positive bands were identical to those detected by an anti-CREB antibody (Fig. 5B). These results suggest that zCaMKIV activated by CaMKK phosphorylates endogenous CREB in the zebrafish brain as well as recombinant rat CREB.

The C-terminal region of CaMKIV modulates the substrate specificities

As shown in Fig. 1, zCaMKIV lacked a region corresponding to the C-terminal region of rCaMKIV. To examine the possibility that the absence of the C-terminal region of CaMKIV affects its substrate specificities, we compared the kinase activity of zCaMKIV with those of rCaMKIV(WT) and rCaMKIV(1-344), which corresponds to zCaMKIV by deletion of the

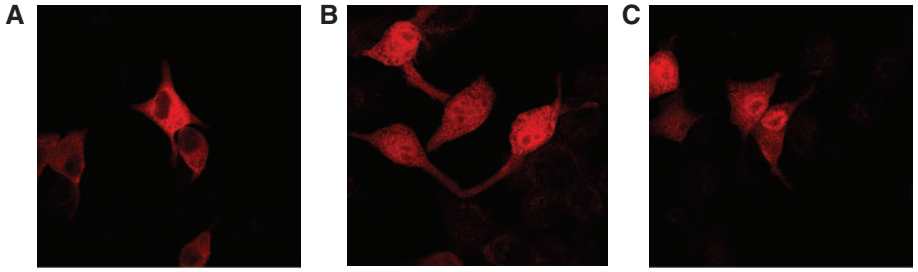

Fig. 3 Subcellular localization of zCaMKIV. Neuro2a cells were transfected with myc-tagged rCaMKIα (A), myc-tagged zCaMKIV (B), or myc-tagged rCaMKIV (C). The transiently expressed myc-tagged proteins were detected by indirect immunofluorescence with an anti-myc antibody and visualized by a confocal laser-scanning microscope.

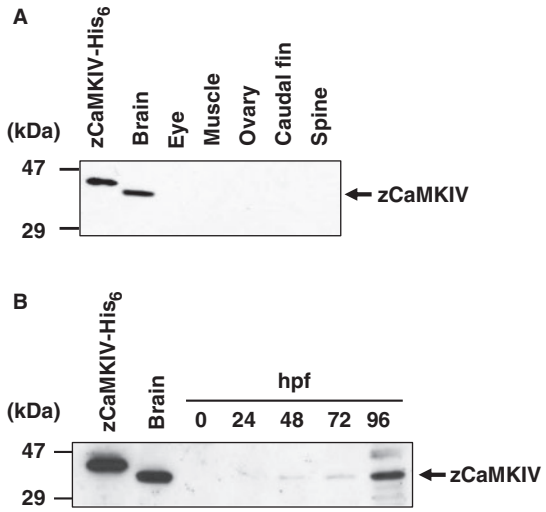

Fig. 4 Tissue distribution and developmental changes of zCaMKIV protein in zebrafish. (A) Crude lysates of various tissues of zebrafish (100 μg), together with recombinant zCaMKIV-His_6 (10 ng), were subjected to western blotting analysis as indicated. The blot membrane was probed with an anti-CaMKIV monoclonal antibody. Molecular weight markers are indicated on the left. (B) Temporal expression of zCaMKIV protein during zebrafish embryogenesis. zCaMKIV was immunoprecipitated with an anti-CaMKIV antibody from crude lysates of adult brain (1 mg protein), embryos harvested at the indicated times (10 mg of protein) and zCaMKIV-His_6 (100 ng). The immunoprecipitated zCaMKIV was separated by SDS-PAGE and analysed by western blotting with DIG-conjugated Multi-PK antibody and an anti-DIG antibody.

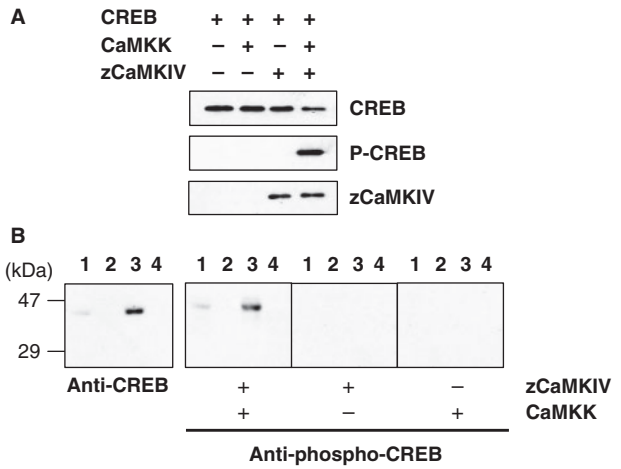

Fig. 5 Phosphorylation of CREB by zCaMKIV. (A) Recombinant rat CREB (50 μg/ml) was incubated in the presence or absence of zCaMKIV (5 μg/ml) or CaMKK (0.5 μg/ml) for 30 min at 30°C in a solution containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP. The reaction was stopped by the addition of 2× SDS-PAGE sample buffer. The samples were then separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-CREB, anti-phospho(Ser-133)-CREB and anti-CaMKIV antibodies. (B) Proteins (25 μg) in a zebrafish brain total extract (lane 1), cytosolic fraction (lane 2), nuclear fraction (lane 3) and membrane/organelle fraction (lane 4) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated for 3 h at 30°C in a reaction mixture containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM ATP with or without 10 μg/ml zCaMKIV and 1 μg/ml CaMKK. The membranes were probed with anti-CREB or anti-phospho(Ser-133)-CREB antibodies.

C-terminal region of rCaMKIV (Fig. 6A). When the substrate specificities were examined using a zebrafish brain extract, the CaMKIVs phosphorylated certain endogenous proteins in the nuclear fraction, and the extents of phosphorylation differed among the CaMKIVs (Fig. 6B, lanes 1 and 4). Judging from its molecular mass and subcellular localization, one of the phosphorylated proteins (Fig. 6B, arrowheads) was thought to be histone H1. To further explore the possibility that the C-terminal region of CaMKIV modulates the substrate specificity, we carried out kinase assays using histone H1, a high molecular mass type of histone, and MBP as substrates. rCaMKIV(WT) showed significantly higher activity towards histone H1 than toward MBP, whereas rCaMKIV(1-344) and zCaMKIV did not (Fig. 6C and D). These results indicate that the C-terminal region of rCaMKIV plays a role in determining its substrate specificity.

Dephosphorylation of CaMKIV and its mutants by CaMKP-N

CaMKIV was reported to be deactivated by protein phosphatases that dephosphorylate a Thr residue located within the activation loop of CaMKIV (*35*). In our previous study (*19*), we showed that the zebrafish homologue of CaMKP-N dephosphorylates rCaMKIV *in vitro*. Therefore, we confirmed that zCaMKP-N also dephosphorylated zCaMKIV as well as rCaMKIV (Fig. 7A and B). Although zCaMKP-N dephosphorylated both rCaMKIV(WT) and zCaMKIV, zCaMKIV was more susceptible to dephosphorylation by zCaMKP-N than rCaMKIV(WT). Since zCaMKIV lacks a region corresponding to the C-terminal region of rCaMKIV, we

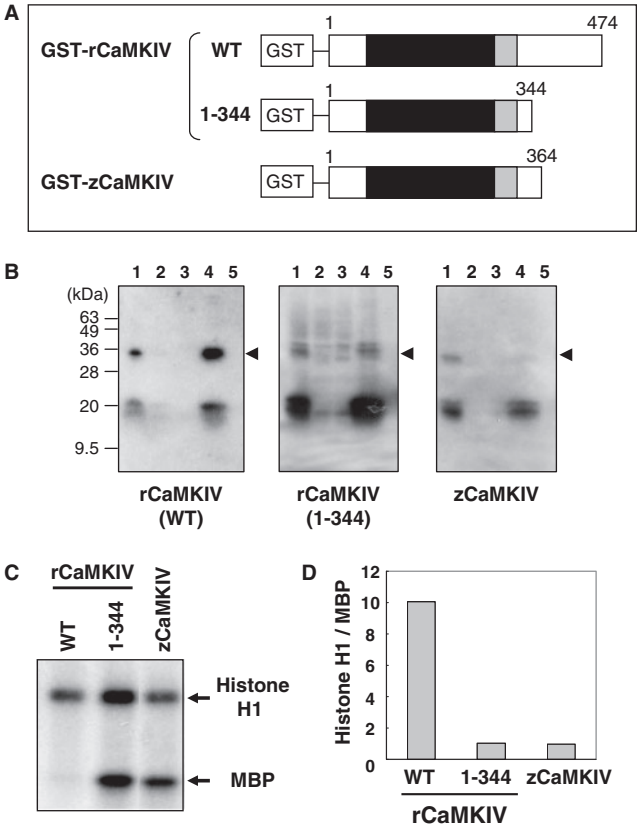

Fig. 6 Modulation of substrate specificity by the C-terminal region of rCaMKIV. (A) Schematic illustrations of the primary structures of GST-fused rCaMKIV(WT), rCaMKIV(1-344), which mimics zCaMKIV by lacking the C-terminal region, and zCaMKIV. The catalytic domain is shaded in black. The autoinhibitory and Ca^{2+}/CaM-binding domain is shaded in gray. (B) Proteins (25 μg) in a zebrafish brain total extract (lane 1), cytosolic fraction (lane 2), membrane/organelle fraction (lane 3), nuclear fraction (lane 4) and cytoskeleton fraction (lane 5) were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated for 4 h at 30°C in a reaction mixture containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM [γ-^{32}P]ATP with 5 μg/ml of GST-rCaMKIV(WT), GST-rCaMKIV(1-344) or GST-zCaMKIV, all of which had been activated by CaMKK. The position of histone H1 was shown by arrowheads. (C) Histone H1 (10 μg/ml) and MBP (10 μg/ml) were incubated in the presence of 2 μg/ml of active GST-CaMKIVs for 10 min at 30°C in a solution containing 50 mM HEPES–NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 1 mM DTT, 1 mM $CaCl_2$, 1 μM CaM and 100 μM [γ-^{32}P]ATP. The reaction was terminated by adding 2× SDS-PAGE sample buffer. The samples were separated by 15% SDS-PAGE and detected by autoradiography. (D) Radioactivities incorporated into histone H1 and MBP were quantitated by Scion Image and represented as ratios of phosphorylation of histone H1/MBP.

speculated that the C-terminal region is responsible for the resistance to dephosphorylation by zCaMKP-N. To examine this possibility, we compared the extent of dephosphorylation of rCaMKIV(1-344), which is devoid of the C-terminal region, with that of rCaMKIV(WT). As expected, rCaMKIV(1-344) was more efficiently dephosphorylated by zCaMKP-N than rCaMKIV(WT) (Fig. 7B). These results suggest that the C-terminal region of rCaMKIV regulates its susceptibility to dephosphorylation by protein phosphatases including zCaMKP-N.

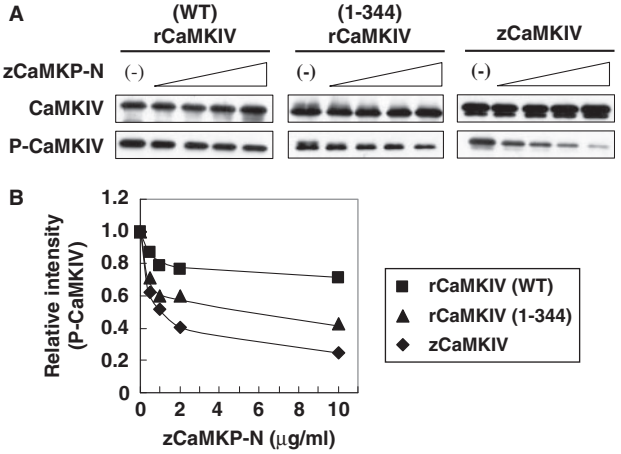

Fig. 7 Dephosphorylation of CaMKIVs by CaMKP-N. (A) Phosphorylated forms (5 μg/ml) of rCaMKIV(WT), rCaMKIV(1-344) and zCaMKIV were dephosphorylated by zCaMKP-N (0.5, 1, 2 or 10 μg/ml) in a reaction mixture containing 50 mM Tris–HCl (pH 8.0), 1 mM DTT and 5 mM $MnCl_2$ for 10 min at 30°C. The reactions were stopped by adding an equal amount of 2× SDS-PAGE sample buffer. The samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-CaMKIV and anti-phospho-CaMKIV antibodies. (B) Dephosphorylation of CaMKIVs by CaMKP-N. Intensity of phosphorylated bands of CaMKIV shown in the lower panel in (A) was quantitated by Scion Image. Phosphorylation level of each band was calculated taking the value without CaMKP-N treatment as 1.0.

Discussion

CaMKIV was first discovered in the rodent brain, and its biochemical properties were elucidated using the rat and human enzymes. In the present study, we focused on the zebrafish homologue of CaMKIV, and found that it was expressed as a shorter form lacking the typical C-terminal region. Although much has been reported about the truncation mutants of CaMKIV, including those devoid of the C-terminal region (*8*,*18*), they are artificially produced enzymes obtained by genetic engineering of mammalian CaMKIV. Since zCaMKIV is composed of regions that are nearly equivalent to only a catalytic domain and an autoinhibitory/CaM-binding domain, it should be the minimum size enzyme with CaMKIV function. Although chicken CaMKIV, which is expressed as a truncated form in spinal cord motoneurons, was reported to show Ca^{2+}-dependent kinase activity towards CREB (*36*), little attention has been given to extensive biochemical characterization of the shorter types of CaMKIV. Here, we report the detailed biochemical properties of zCaMKIV, a shorter type CaMKIV homologue with the minimum size able to function as CaMKIV.

We concluded that the cDNA clone obtained in the present study was that of the zebrafish homologue of CaMKIV based on the following biochemical observations: (i) the predicted amino acid sequence was highly homologous to rCaMKIV; (ii) it bound CaM in a Ca^{2+}-dependent manner; (iii) it was strongly activated by CaMKK-catalysed phosphorylation of a conserved Thr residue located in the activation loop; (iv) the

phosphorylated kinase was effectively dephosphorylated by CaMKP-N; (v) it was predominantly expressed in the brain and mainly localized in the nuclei; and (vi) it phosphorylated Ser-133 of CREB in a CaMKK-dependent manner. All of these lines of evidence strongly suggested that the cloned enzyme functioned as CaMKIV in the zebrafish brain. The present findings also indicate that the C-terminal region of rCaMKIV is not essential for CaMKIV function, because zCaMKIV lacks a C-terminal region corresponding to amino acids 345–474 of rCaMKIV.

Little is known about the function of the C-terminal region of rCaMKIV. In an attempt to clarify this function, we compared the substrate specificity of a C-terminal deletion mutant of rCaMKIV with that of the zebrafish enzyme, a naturally occurring 'truncation mutant'. The wild-type rCaMKIV, deletion mutant rCaMKIV(1-344) and zCaMKIV phosphorylated CREB efficiently (data not shown). However, rCaMKIV preferred histone H1 to MBP, whereas rCaMKIV(1-344) and zCaMKIV did not. These findings suggest that the C-terminal region of CaMKIV plays some important but still unknown roles in regulating its substrate recognition.

In addition, we examined whether CaMKP-N catalysed the dephosphorylation of these kinases after they had been phosphorylated by CaMKK. We found that zCaMKP-N dephosphorylated zCaMKIV more efficiently than rCaMKIV. Unlike rCaMKIV, however, the C-terminal truncation mutant of rCaMKIV that mimics zCaMKIV also served as an efficient substrate for zCaMKP-N. These results suggest that the C-terminal region of CaMKIV plays a role in regulating its recognition as a substrate for CaMKP-N. Therefore, it is likely that the C-terminal region is involved in the regulation of both its substrate specificity and competence for dephosphorylation by CaMKP-N.

CaMKIV is predominantly localized in nuclei (*33*). Lemrow *et al.* (*37*) reported that the protein kinase domain of CaMKIV is necessary and sufficient for nuclear entry of CaMKIV. This implies that the N-terminal Ser-rich region and the C-terminal domain containing the nuclear localization sequence are not required for nuclear entry. This is in good agreement with our finding that zCaMKIV, which was devoid of the C-terminal domain, was localized in the nuclei of Neuro2a cells. Although the molecular mechanism for the nuclear entry of mammalian CaMKIVs has not been fully elucidated, an interaction with importin α has been proposed to be involved (*38*). The aspect of whether the mechanism of nuclear entry of zCaMKIV is similar to that of mammalian CaMKIVs remains to be clarified. On the other hand, we found that a considerable amount of zCaMKIV also seemed to exist in the cytosol. It may arise from a decrease in acidity resulting from lacking of the C-terminal domain, because zCaMKIV has a somewhat higher predicted p*I* value than rCaMKIV. In our previous report (*19*), we assumed that the developmental abnormalities induced by anti-sense knockdown of CaMKP-N were caused by aberrant activation of CaMKIV in the zebrafish brain. In the present study, we have shown that zCaMKIV is predominantly expressed in the brain, being in good agreement with the tissue distributions of mammalian CaMKIVs.

We also investigated the developmental changes in zCaMKIV during embryogenesis, and found that zCaMKIV expression was markedly increased after 72 hpf. In adult brain, CaMKIV is known to be abundantly expressed in cerebellar granule cells in mammals (*5*). In zebrafish, granule cells as well as Purkinje cells begin to differentiate at 72–90 hpf (*39*). The granule cells are located beneath the Purkinje cell layer, and the Purkinje cells extend their dendrites and receive inputs from the parallel fibres of the granule cells at around 120 hpf. These facts implicate that the cerebellar neurons and neural circuits significantly develop between 72 and 120 hpf. Therefore, it is reasonable to speculate that CaMKIV plays important roles in the development of the zebrafish brain during this stage. It will be interesting to clarify why CaMKIV needs to be expressed as the shorter form in zebrafish in the future studies.

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Conflict of interest

None declared.

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