J. Biochem. **135**, 619–630 (2004)
DOI: 10.1093/jb/mvh075

Cloning and Characterization of a Novel Ca^{2+}/Calmodulin-Dependent Protein Kinase I Homologue in *Xenopus laevis*

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Received January 23, 2004; accepted March 15, 2004

In order to investigate protein kinases expressed in the different developmental stages of *Xenopus laevis*, recently developed expression cloning was carried out. When two different expression libraries, *Xenopus* oocyte and *Xenopus* head (embryonic stage 28/30) cDNA libraries, were screened by kinase-specific monoclonal antibodies, cDNA clones for various known and novel protein serine/threonine kinases (Ser/Thr kinases) were isolated. In addition to well-characterized Ser/Thr kinases, one cDNA clone for a putative kinase was isolated from the *Xenopus* head library. The sequence of the open reading frame of the cDNA encoded a protein of 337 amino acid residues with a predicted molecular weight of 38,404. Since the deduced animo acid sequence of this protein was 75% identical to that of rat Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), it was designated as CaMKIx. Although recombinant CaMKIx expressed in *Escherichia coli* showed no protein kinase activity against syntide-2, a synthetic peptide substrate, it was activated when phosphorylated by mouse Ca^{2+}/calmodulin-dependent protein kinase kinase α (CaMKKα). Activated CaMKIx significantly phosphorylated various proteins including synapsin I, histones, and myelin basic protein. CaMKIx could not be detected in the early stages of embryogenesis, but was detected in late embryos of stages 37/38 and thereafter when examined by Western blotting using a specific antibody. This kinase was found to be highly expressed in adult brain and heart, and an upstream kinase that could activate CaMKIx was detected in these tissues. These results suggest that CaMKIx plays some critical role in the late stages of embryogenesis of *Xenopus laevis*.

Key words: CaM-kinase, embryogenesis, expression cloning, Ser/The kinase, *Xenopus laevis*.

Abbreviations: CaM, calmodulin; CaMKKα, Ca^{2+}/calmodulin-dependent protein kinase kinase α; CaMKIx, *Xenopus* Ca^{2+}/calmodulin-dependent protein kinase I; CaMKPase, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; MLC, myosin light chain; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Ser/Thr kinase, protein serine/threonine kinase.

Protein kinases are known to play pivotal roles in various signaling pathways and to participate in diverse cellular processes. The eukaryotic protein kinases consist of large families of homologous proteins, comprising 1.5–2.5% of all gene products, that can be classified into almost 200 subfamilies on the basis of kinase domain similarity (*1*). Although hundreds of protein kinases have been documented to date, it is still unclear how many and what kinds of protein kinases are expressed in cells and tissues under varying situations. In order to detect a wide variety of protein kinases expressed in various cells and tissues, we developed a unique expression cloning technique to isolate cDNA clones for various protein kinases using monoclonal antibodies directed to highly conserved regions of protein kinases (*2*). Using this technique, we isolated various known and novel protein kinases expressed in mouse brain (*2*).

During oogenesis and embryogenesis of *Xenopus laevis*, various protein kinases are believed to be expressed in cell-cycle specific and stage-specific manners and may play critical roles in these processes. Early developmental stages are known to be programmed by maternally inherited mRNA and proteins. By differential screening of a *Xenopus laevis* egg cDNA library, Paris and Philippe isolated 11 cDNA clones (*3*) that appeared to be essential for early embryonic development. Several proteins corresponding to these clones have been characterized; pEg1 (*4*), pEg2 (*5*), and pEg3 (*6*) were identified as novel protein kinases that correlate with cell-cycle regulation. Although a variety of protein kinases have been reported to be expressed during different developmental stages of *Xenopus laevis*, there may be novel protein kinases still remaining to be elucidated.

For the detection and isolation of protein kinases that correlate with embryogenesis of *Xenopus laevis*, we attempted to screen protein kinases expressed in *Xenopus* oocytes and/or embryos. In this study, a cDNA clone for a novel protein kinase exhibiting 75% identity to that

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of rat Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) was obtained from a *Xenopus* head library by screening with kinase-specific antibodies. The expression of *Xenopus* CaMKI (CaMKIx) was found to be stage-specific as revealed by Western blotting with a CaMKIx-specific antibody, suggesting that CaMKIx may play some roles in the process of embryogenesis of *Xenopus laevis*.

MATERIALS AND METHODS

Materials—ATP, bovine serum albumin, histone type IIA from calf thymus, α-casein from bovine milk, and myelin basic protein (MBP) from bovine brain were purchased from Sigma Chemicals. Goat anti-mouse IgG and goat anti-rabbit IgG, conjugated with horseradish peroxidase, were obtained from ICN Pharmaceuticals. [γ-^{32}P]ATP (5,000 Ci/mmol) and a HiTrap Chelating HP column were from Amersham Biosciences. Syntide-2 and Kemptide were synthesized using a Shimadzu PSSM-8 automated peptide synthesizer, and purified by reverse-phase HPLC on a C_{18} column (ODS-80Tm, Tosoh). Smooth muscle myosin light chain (MLC), microtubule-associated protein 2 (MAP2), and synapsin I were prepared as described previously (*7*). Calmodulin (CaM) was purified from rat testis as described previously (*8*). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart (*9*).

Two monoclonal antibodies, M8C and M1C, were obtained from two hybridoma cell lines established as described previously (*2*).

A *Xenopus* oocyte λTriplEx cDNA library was obtained from CLONTECH and a stage 28/30 (tailbud tadpole) head library in the λZAPII library was kindly provided by Dr. Richard Harland, University of California, Berkeley.

Construction of Plasmid, Expression, Purification of CaMKIx, CaMKKα and CaMKPase—In the case of CaMKIx, the following primers were used for PCR: CaMKIx-5′ upstream primer (5′-TTA GCT AGC ATG CCT CTT GGG AAG AAC GTC AA-3′) and CaMKIx-3′ downstream primer (5′-ACT CGA GCC ACT TGT CCT TCT CTT CTG GAA-3′). The CaMKIx-5′ upstream primer contained an *Nhe*I restriction site (underlined) and the CaMKIx-3′ downstream primer contained an *Xho*I site (double underlined). In the case of CaMKKα, an insert that included the full-length of the open reading frame with *Nhe*I and *Sal*I sites was amplified using a set of primers: CaMKKα-5′ upstream primer (5′-GTC GCT AGC ATG GAG AGT GGC CCA GCC GT-3′) and CaMKKα-3′ downstream primer (5′-TTT GTC GAC GGA CGC AGC CTC ATC CTC CT-3′). The CaMKKα-5′ upstream primer contained an *Nhe*I restriction site (underlined) and the CaMKKα-3′ downstream primer contained a *Sal*I site (double underlined). PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles (each consisting of denaturation at 96°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 2 min) using Pyrobest DNA Polymerase (TaKaRa), XHZ049 or a mouse brain 5′-RACE ready cDNA library as a template. After gel purification, the amplified product was digested with *Nhe*I and *Xho*I (or *Nhe*I and *Sal*I). To generate the His_6-tag fusion proteins, the *Nhe*I/*Xho*I (or *Nhe*I/*Sal*I) fragment was cloned into the *Nhe*I/*Xho*I (or *Nhe*I/*Sal*I)-digested pET-23a(+) (Novagen), sequenced, and designated as pETCaMKIx or pETCaMKKα.

BL21(DE3) cells transformed with pETCaMKIx (or pETCaMKKα) were grown at 37°C for 16 h in 3 ml of medium A (LB medium supplemented with 100 μg/ml ampicillin) with shaking. The culture was then transferred to a 300-ml flask containing 100 ml of medium A and incubated at 25°C for 24 h (in the case of CaMKIx) or 37°C for 18 h (in the case of CaMKKα) with shaking. The cells were harvested by centrifugation, and suspended in 10 ml of buffer A [20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.05% Tween 40]. After sonication, cell debris was removed by centrifugation (20,000 ×*g*) at 4°C for 10 min, and the supernatant obtained was loaded on a HiTrap Chelating HP column (1 ml) pre-equilibrated with buffer A. The column was washed successively with 10 ml of buffer A, buffer A containing 20 mM imidazole, and buffer A containing 50 mM imidazole, and then CaMKIx (or CaMKKα) was eluted with buffer A containing 200 mM imidazole. The active fractions were pooled and stored in aliquots at –80°C until use.

His_6-tagged wild type Ca^{2+}/calmodulin-dependent protein kinase phosphatase (CaMKPase) was prepared essentially as described previously (*10*).

Preparation of Crude Extracts from Xenopus Eggs, Embryos, and Tissues—Unfertilized eggs were obtained from female *Xenopus laevis* by injecting 300 units of Gonatropin (Teikoku Hormone MFG.) 14–16 h prior to the experiment. For embryos, eggs were fertilized *in vitro*; dejellied in 2% cysteine (pH 7.5), washed in 0.1× Steinberg's solution [1 mM Hepes-NaOH (pH 7.4), 6 mM NaCl, 67 μM KCl, 83 μM $MgSO_4$, and 34 μM $Ca(NO_3)_2$], and cultured at 22°C in 0.1× Steinberg's solution. The embryos were staged according to Nieuwkoop and Faber (*11*). Embryos were collected at the indicated stages, frozen in liquid nitrogen, and stored frozen at –80°C until further analysis.

Crude extracts of oocytes and embryos of the indicated stages were prepared as follows. Eggs and embryos were suspended in 3 volumes of homogenizing buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.05% Tween 40, and homogenized in a Teflon/glass homogenizer. Crude extracts from brain, heart, spleen, liver, muscle, and ovary were prepared as above except that 5 volumes of homogenizing buffer were used and the tissues were homogenized with a Polytron (KINEMATICA AG). The homogenates were centrifuged at 20,000 ×*g* at 2°C for 30 min, and the supernatants obtained were used as crude extracts.

Protein Determination—The concentrations of synapsin I and CaM were determined spectrophotometrically using $E_{1\%}$ = 6.74 at 277 nm (*12*) and $E_{1\%}$ = 1.8 at 276 nm (*13*), respectively. Other protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard (*14*).

Cloning of Protein Kinases Expressed in Xenopus laevis—Two cDNA libraries, a *Xenopus* oocyte λTriplEx cDNA library and a stage 28/30 (tailbud tadpole) λZAPII library, were used to screen protein kinases expressed in different stages of development of *Xenopus laevis*. Screening of the *Xenopus* cDNA expression libraries was carried out using monoclonal antibodies directed to highly conserved regions in protein kinase family enzymes (*2*).

Cloning, sequencing, and data analyses of cDNA clones corresponding to various protein kinases were carried out as described previously (*2*).

Production of Antibody Directed to CaMKIx—Antibodies were produced in 8-week old Japanese white rabbits (Japan SLC). Approximately 200 μg of a purified preparation of recombinant CaMKIx, emulsified with an equal volume of Freund's complete adjuvant (DIFCO Laboratories), was injected at multiple intradermal sites, followed two weeks later by an injection of the same dosage of CaMKIx emulsified in Freund's incomplete adjuvant (DIFCO Laboratories). Two intravenous boosters of 100 μg each of CaMKIx in phosphate-buffered saline were given to rabbits at 2-week intervals. Antisera were harvested one week after the final injection and stored in aliquots at –80°C until use.

SDS-PAGE and Western Blotting—SDS-PAGE was carried out essentially according to the method of Laemmli (*15*) on slab gels consisting of an 8%, 10% or 12% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to a nitrocellulose membrane (Protran BA85, Schleicher & Schuell) and incubated with an antiserum at a dilution of 1:1,000 in 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20. Membranes were then treated with anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000 dilution), and immuno-reactive protein bands were visualized by the chemiluminescent substrate, SuperSignal West Dura Extended Duration Substrate (Pierce) as described previously (*2*).

Biotinylated-CaM Overlay Assay—CaM was biotinylated using NHS-Biotin (Sigma Chemicals) essentially according to the method described previously (*16*, *17*). The biotinylated-CaM overlay assay was carried out as follows: Protein samples were resolved on SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated in washing buffer [50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.05% Tween 20] containing 5% skim milk for 1 h at room temperature, and then washed three times with washing buffer containing either 1 mM $CaCl_2$ or 1 mM EGTA. The membranes were incubated with biotinylated-CaM (0.7 μg/ml) in washing buffer containing 1 mM $CaCl_2$ or 1 mM EGTA at room temperature for 1 h. After the membranes were rinsed three times with washing buffer in the presence of 1 mM $CaCl_2$ or 1 mM EGTA, they were incubated with avidin conjugated with horseradish peroxidase (Sigma Chemicals) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA at room temperature for 1 h. The membranes were washed twice with washing buffer and then twice with washing buffer without Tween 20, each containing either 1 mM $CaCl_2$ or 1 mM EGTA. CaM binding proteins were visualized using a chemiluminescent substrate, SuperSignal West Dura Extended Duration Substrate.

Protein Kinase Assay—The protein kinase activity of CaMKIx was determined essentially according to the method described previously (*18*). Phosphorylation was carried out at 30°C in a final volume of 20 μl in a reaction mixture consisting of 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 40 μM syntide-2, 100 μM [γ-^{32}P]ATP (300–500 cpm/pmol), and the indicated amount of CaMKIx in the presence or absence of 0.5 mM $CaCl_2$/1 μM CaM. After incubation at 30°C for 1, 2, and 3 min, a 5-μl aliquot of the mixture was withdrawn, spotted onto a 2-cm square of phosphocellulose paper P81 (Whatman), and immediately placed in 75 mM phosphoric acid. The ^{32}P-phosphate incorporation into syntide-2 was measured essentially according to the method of Roskoski (*19*). When proteins were used as substrates, the incorporation of ^{32}P-phosphate into the substrate was measured by the Whatman 3MM chromatography paper method of Corbin and Reimann (*20*).

For the activation of CaMKIx, the enzyme was phosphorylated by mouse recombinant CaMKKα. Purified CaMKIx (2 μg) was incubated for 20 min at 30°C with CaMKKα (0.1 μg) in a reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 100 μM ATP, 0.5 mM $CaCl_2$, and 1 μM CaM in a final volume of 20 μl. The reaction was stopped by the addition of 80 μl of stop buffer containing 50 mM Tris-HCl (pH 7.5), 0.05% Tween 40, 2 mM dithiothreitol, and 2.5 mM EGTA. CaMKKα-activated CaMKIx was stored in aliquots at –80°C before use.

In-Gel Protein Phosphatase Assay—Protein phosphatase activities in *Xenopus* crude extracts were detected by in-gel protein phosphatase assay (*21*) using ^{32}P-phosphorylated protein substrates. CaMKIx (5 μg) was phosphorylated in a reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 50 μM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, 1 μM CaM, and 0.25 μg CaMKKα in a final volume of 50 μl at 30°C. MBP (5 μg) was also phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (0.25 μg) as above except that Ca^{2+}/CaM and CaMKKα were omitted from the reaction mixture. After 20 min of incubation, 0.25 μg CaMKKα (or cAMP-dependent protein kinase) was added again, and the samples were incubated at 30°C for additional 30 min. The reactions were stopped by adding EGTA to a final concentration of 10 mM. Protein substrates, thus phosphorylated, were added to 10% acrylamide solutions (1×10^6 cpm/gel) just prior to polymerization. Crude extracts from various tissues of adult *Xenopus* were resolved in SDS-polyacrylamide gels containing phosphorylated protein substrates; the resolved proteins were renatured *in situ*, and protein phosphatase activities detected essentially as described previously (*21*).

RESULTS

Expression Cloning of Protein Kinases in Xenopus laevis—In the previous study, we developed a unique expression cloning technique using monoclonal antibodies directed to highly conserved sequences (subdomain VIB) in protein kinases, and isolated cDNA clones for various Ser/Thr kinases in mouse brain (*2*). In this study, we employed this technique to explore protein kinases expressed in *Xenopus laevis*.

When a *Xenopus* oocyte cDNA expression library was screened with kinase-specific antibodies, 18 positive clones were obtained from 4.9×10^5 plaques (Table 1). The positive clones thus obtained were sequenced and analyzed by BLAST homology search, revealing them to be well-known Ser/Thr kinases such as CaMKII, cAMP-dependent protein kinase, checkpoint kinase, and egg-specific Ser/Thr kinases such as p46Eg265 protein (*5*),

Table 1. **Identity of cDNA clones isolated from a *Xenopus* oocyte expression library.** Eighteen positive clones obtained from a *Xenopus* oocyte λTriplEx cDNA library by immunoscreening with monoclonal antibodies were isolated, sequenced, and analyzed by a BLAST homology search.

Clone	Identified protein	Accession No.	Recognition sequence[a]
XET003	CaMKII-γ M subunit	AF233633	IVHRDLKPENLLLAS
XET004	p46Eg265 protein	Z17207	VIHRDIKPENLLLGS
XET008	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XET011	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XET012	p69Eg3 protein	Z17205	YAHRDLKPENLLIDE
XET013	p46Eg265 protein	Z17207	VIHRDIKPENLLLGS
XET014	CaMKII-γ J subunit	AF233630	IVHRDLKPENLLLAS
XET016	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XET017	CaMKII-γ J subunit	AF233630	IVHRDLKPENLLLAS
XET018	p46Eg265 protein	Z17207	VIHRDIKPENLLLGS
XET023	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XET026	Chk1 checkpoint kinase	AF117816	ITHRDIKPENLLLDE
XET029	p69Eg3 protein	Z17205	YAHRDLKPENLLIDE
XET030	p69Eg3 protein	Z17205	YAHRDLKPENLLIDE
XET035	p46Eg265 protein	Z17207	VIHRDIKPENLLLGS
XET036	p46Eg265 protein	Z17207	VIHRDIKPENLLLGS
XET038	PKA	AJ413218	LIYRDLKPENLLIDQ
XET042	Similar to CaMKII-γ	BC049002	IVHRDLKPENLLLAS

[a]Amino acid sequence corresponding to possible antigenic sites.

Table 2. **Identity of cDNA clones isolated from a *Xenopus* tadpole expression library.** Fourteen positive clones obtained from a *Xenopus* tadpole λZAPII cDNA library by immunoscreening with monoclonal antibodies were isolated, sequenced, and analyzed by a BLAST homology search.

Clone	Identified protein	Accession No.	Recognition sequence[a]
XHZ002	Phosphotyrosyl-protein phosphatase	U15287	[b]
XHZ003	CaMKII-γ M subunit	AF233633	IVHRDLKPENLLLAS
XHZ005	CaMKII-γ K subunit	AF233631	IVHRDLKPENLLLAS
XHZ006	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XHZ007	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XHZ009	p46XlEg22 protein	Z17206	VIHRDIKPENLLLGS
XHZ010	Chk1 checkpoint kinase	AF117816	ITHRDIKPENLLLDE
XHZ033	Chk1 checkpoint kinase	AF117816	ITHRDIKPENLLLDE
XHZ037	CaMKII-γ L subunit	AF233632	IVHRDLKPENLLLAS
XHZ038	CaMKII-γ L subunit	AF233632	IVHRDLKPENLLLAS
XHZ039	Chk1 checkpoint kinase	AF117816	ITHRDIKPENLLLDE
XHZ042	CaMKII-γ M subunit	AF233633	IVHRDLKPENLLLAS
XHZ048	CaMKII-γ J subunit	AF233630	IVHRDLKPENLLLAS
XHZ049	CaMKI homologue	AB098710[c]	IVHRDLKPENLLYAT

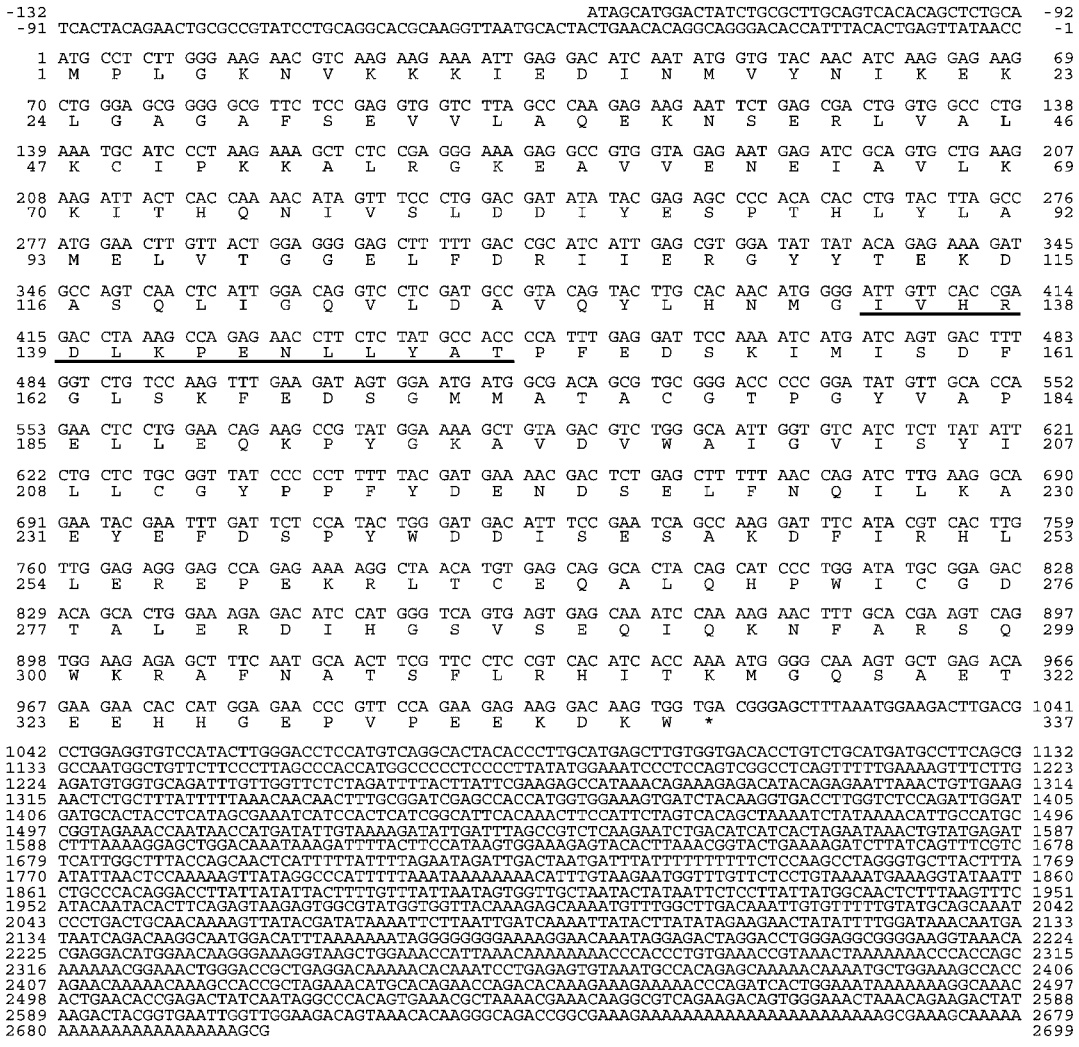
[a]Amino acid sequence corresponding to possible antigenic sites. [b]Phosphotyrosyl-protein phosphatase is a non-kinase protein that has no possible recognition sequence. [c]Accession number obtained in this study.

p46XlEg22 protein (*22*), and p69Eg3 protein (*6*). When a *Xenopus* tailbud tadpole (stage 28/30) head library was screened, 14 positive clones were obtained from 3.7×10^5 plaques (Table 2). Among the 14 positive clones isolated from the tadpole head library, 12 clones were identified as Ser/Thr kinases such as CaMKII, checkpoint kinase, and p46XlEg22 protein, and one clone was identified as phosphotyrosyl-protein phosphatase (Table 2). Since the nucleotide sequence or predicted amino acid sequence of clone XHZ049 was not found in the database, this cDNA was thought to encode a novel protein kinase not reported previously.

Identification of a Novel Protein Kinase Expressed in a Tailbud Tadpole Head Library—Since the cDNA clone (XHZ049) isolated from the tailbud tadpole head cDNA library was found to be a novel protein kinase, the clone was further characterized. The whole nucleotide sequence of this clone and its deduced amino acid sequence

Fig. 1. **Sequence analyses of the *Xenopus* XHZ049 gene.** A, nucleotide and deduced amino acid sequences of XHZ049. A predicted open reading frame of 337 amino acids is shown under the nucleotide sequence. These nucleotide sequence data are available from the DDBJ under the accession number AB098710. The underlined sequence represents the possible recognition site of the monoclonal antibody used for immunoscreening. B, alignment of the deduced amino acid sequence of the gene product with rat CaMKIβ2 (*24*). Identical amino acids are shaded in black. Twelve subdomains specific to protein kinases (*23*) are underlined. Broken underline represents the possible regulatory domain (*28*); Thr174 indicated by an asterisk corresponds to the possible phosphorylation site for activation.

A



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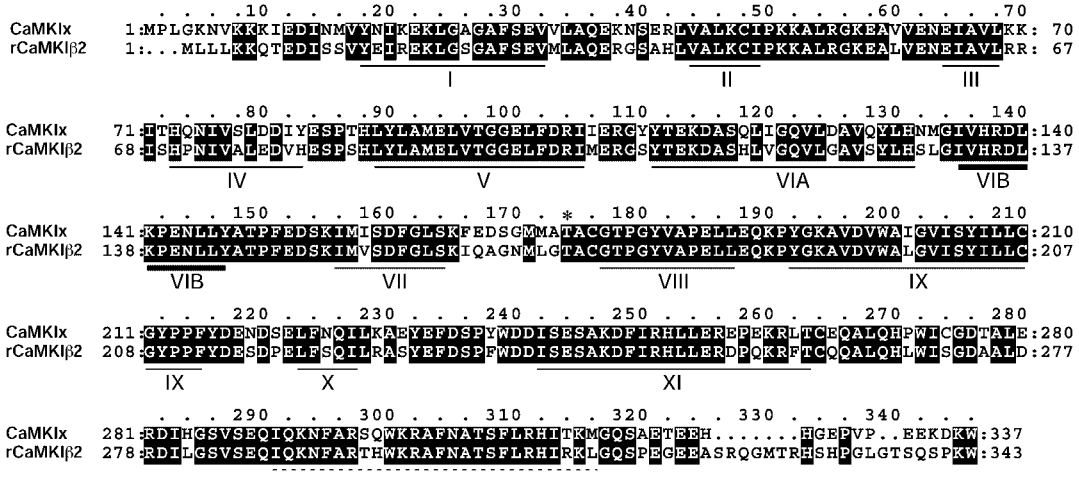

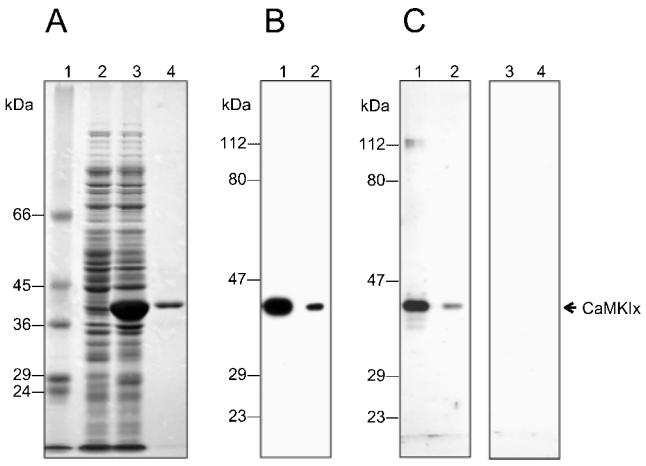

Fig. 2. **Expression and purification of recombinant CaMKIx and electrophoretic analyses.** *A*, expression of recombinant CaMKIx in *E. coli* cells. Crude extract (17.5 μg protein, lane 3) of *E. coli* cells transformed with pETCaMKIx and mock-transfected *E. coli* extract (10 μg protein, lane2) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Recombinant CaMKIx (0.5 μg) purified on a HiTrap Chelating HP column is also shown (lane 4). The marker proteins (lane 1) used were bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), and trypsinogen (M_r 24,000). B, Western blotting with a kinase-specific monoclonal antibody. Purified CaMKIx (10 ng; lane 1, 1 ng: lane 2) was resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and detected by Western blotting with monoclonal antibody M8C, which was used for immunoscreening in this study, as described under "MATERIALS AND METHODS." C, purified CaMKIx (10 ng; lanes 1 and 3, 1 ng; lanes 2 and 4) were resolved on SDS-PAGE, blotted onto a nitrocellulose membrane, and a biotinylated-CaM overlay experiment was carried out in the presence of 1 mM $CaCl_2$ (lanes 1 and 2) or 1 mM EGTA (lanes 3 and 4). The arrow to the right indicates the migration position of recombinant CaMKIx.

are shown in Fig. 1A. The open reading frame of the gene encodes a polypeptide consisting of 337 amino acid residues with a predicted molecular weight of 38,404. An antigenic recognition sequence, IVHRDLKPENLLYAT, which corresponds to subdomain VIB of protein kinases, was found in the middle part of the sequence (Fig. 1A, underline). Furthermore, the predicted open reading frame contains all of the 12 highly conserved subdomains (Fig. 1B) characteristic of protein kinases (*23*), suggesting that this cDNA clone encodes a protein kinase. When BLAST homology search was carried out, the amino acid sequence of this putative protein kinase was found to share 75% identity with that of the rat CaMKIIβ2 isoform (*24*) (Fig. 1B). Since the open reading frame of XHZ049 was found to encode a CaMKI homologue from *Xenopus laevis*, we designated it as CaMKIx.

Expression and Characterization of CaMKIx—In order to characterize CaMKIx further, the coding sequence of XHZ049 was subcloned into the pET-23a(+) bacterial expression vector to construct pETCaMKIx. The pET-CaMKIx expression vector was transfected into *E. coli* strain BL21(DE3), and the carboxyl-terminal His_6-tagged protein was expressed. As shown in Fig. 2A, the recombinant CaMKIx was expressed in large amounts in a soluble form, and could readily be purified using a nickel affinity column. The purified protein appeared as a single band corresponding to a molecular mass of 39 kDa on SDS-PAGE (Fig. 2A, lane 4). This protein could be immunostained with the monoclonal antibody, M8C, which is used to screen expression libraries to detect positive clones for protein kinases (Fig. 2B). A CaM-overlay experiment was carried out to examine whether the recombinant protein is really a CaM-binding protein. As shown in Fig. 2C, biotinylated-CaM bound to the 39-kDa protein band when incubated in the presence of 1 mM $CaCl_2$ but not in the presence of 1 mM EGTA, suggesting that this protein is a member of the CaM-dependent family of protein kinases.

Next, we examined the protein kinase activity of the recombinant CaMKIx. When the protein kinase activity of CaMKIx was measured using a peptide substrate, syntide-2, almost no protein kinase activity was detected either in the presence or absence of Ca^{2+}/CaM (not shown), suggesting that the purified recombinant protein is an inactive enzyme.

Mammalian CaMKI and CaMKIV are known to be activated by an upstream kinase, CaMKK (*25–28*). Therefore, in order to examine whether CaMKIx can be phosphorylated by CaMKK, CaMKIx was incubated with mouse recombinant CaMKKα in the presence or absence of Ca^{2+}/CaM. As shown in Fig. 3A, CaMKIx was significantly phosphorylated by CaMKKα only in the presence of Ca^{2+}/CaM (Fig. 3A, lane 4*)*. After 20 min of phosphorylation, approximately one mol of phosphate was incorporated into one mol of CaMKIx (Fig. 3B), suggesting that one major site on the protein can be phosphorylated.

The protein kinase activity of CaMKIx toward various protein substrates was examined in reaction mixtures containing either Ca^{2+}/CaM and/or CaMKKα. As shown in Fig. 4, MBP was significantly phosphorylated when CaMKIx was incubated in the presence of Ca^{2+}/CaM, CaMKKα, and MBP (Fig. 4, lane 8). MBP phosphorylation was not significant when any one of Ca^{2+}/CaM, CaMKIx, or CaMKKα was omitted from the phosphorylation mixture (Fig. 4, lanes 5–7). Synapsin I and histones were also phosphorylated significantly in the presence of Ca^{2+}/CaM, CaMKIx, and CaMKKα (Fig. 4, lanes 12 and 20). Although MLC and MAP2 also served as good substrates for CaMKIx (Fig. 4, lanes 4 and 24), casein was found to be a poor substrate for CaMKIx (Fig. 4, lane 16).

The protein kinase activity of phosphorylated CaMKIx was determined using peptide and protein substrates. As shown in Table 3, syntide-2 served as an efficient substrate for the activated CaMKIx, and the phosphorylation of syntide-2 was solely dependent on Ca^{2+}/CaM. Kemptide, which is an efficient substrate for cAMP-dependent protein kinase, was not phosphorylated by CaMKIx. MBP and Synapsin I were found to be effectively phosphorylated by the activated CaMKIx in the presence of Ca^{2+}/CaM. MLC, histones, and MAP2 also served as substrates for CaMKIx, but casein was not phosphorylated (Table 3). These results, taken together, suggest that CaMKIx is a multifunctional protein kinase that may be activated by an upstream CaMKK in *Xenopus laevis*.

Expression of CaMKIx in Xenopus laevis—The tissue distribution of CaMKIx was examined by Western blotting using a specific antibody raised against recombinant CaMKIx. Tissue extracts of adult brain, medulla oblon-

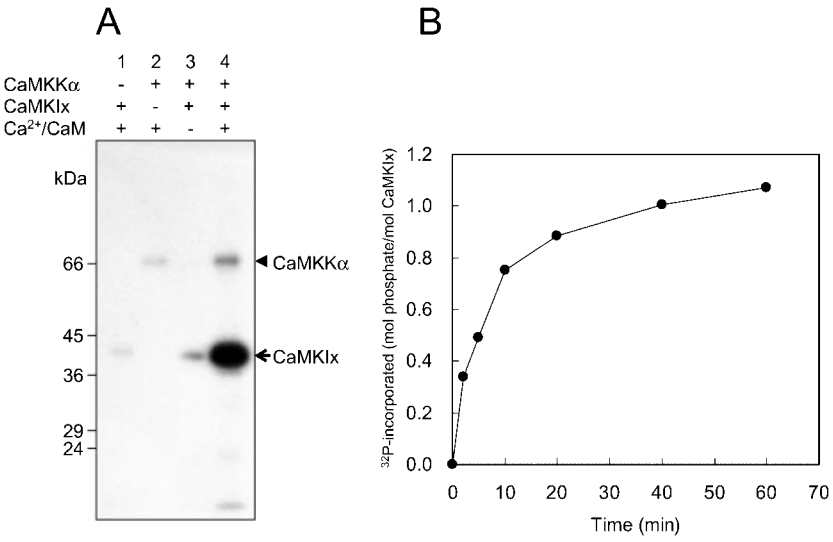

Fig. 3. **Phosphorylation of CaMKIx by mouse CaMKKα.** A, phosphorylation of CaMKIx by mouse CaMKKα. Purified preparations of CaMKIx (4 μg, lane 1) and CaMKKα (0.2 μg, lane 2) were incubated in a reaction mixture consisting of 40 mM Hepes-NaOH (pH 8.0), 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $Mg(CH_3COO)_2$, 100 μM [γ-^{32}P]ATP, 0.5 mM $CaCl_2$, and 1 μM CaM in a final volume of 40 μl for 20 min at 30°C. Mixtures of CaMKIx (4 μg) and CaMKKα (0.2 μg) were incubated either in the absence (lane 3) or presence (lane 4) of Ca^{2+}/CaM as above. A 2.5-μl aliquot was removed from each reaction mixture and mixed with the same volume of SDS sample buffer. The proteins resolved by SDS-PAGE were analyzed by autoradiography. The migration positions of CaMKIx and CaMKKα are shown by the arrow and arrowhead, respectively, on the right of the autoradiogram; the positions of the molecular size markers are shown on the left. B, time course of CaMKIx phosphorylation. Purified CaMKKα was incubated with Ca^{2+}/CaM in the presence or absence of CaMKIx as in (A). From the reaction mixtures, 2.5 μl of aliquots were removed at the indicated times, spotted on Whatman 3MM paper, and phosphate incorporation into CaMKIx was measured in a liquid scintillation counter as described under "MATERIALS AND METHODS." Phosphate incorporated into CaMKIx was calculated as mol phosphate/mol CaMKIx by subtracting radioactivity into [CaMKKα + Ca^{2+}/CaM] from that into [CaMKIx + CaMKKα + Ca^{2+}/CaM], assuming the molecular weight of CaMKIx to be 38,000.

gata, heart, spleen, liver, ovary, skeletal muscle, and unfertilized egg were prepared from *Xenopus laevis*, resolved on SDS-PAGE, and analyzed by Western blotting. The protein staining and immunostaining patterns of *Xenopus* tissue extracts are shown in Fig. 5, A and B, respectively. CaMKIx, which migrated slightly faster than His_6-tagged recombinant CaMKIx, was clearly detected in the brain, medulla oblongata, and heart extracts (Fig. 5B, lanes 1–4). It was also slightly detected in spleen, but not in other tissues examined (Fig. 5B, lanes 5–9).

Although CaMKIx was not detected in unfertilized eggs, it was clearly detected in some tissues of adult *Xenopus laevis* (Fig. 5B), suggesting that the expression of this protein increases at some stage during embryogenesis. Therefore, we examined the changes of expression of CaMKIx during the developmental stages of embryogenesis using extracts from different embryonic stages of *Xenopus laevis*. The protein staining and immunostaining patterns of extracts from embryos at various stages are shown in Fig. 5, C and D, respectively. CaMKIx could not be detected in extracts of embryonic stages before 25/27, but was slightly detected in the extract of embryonic stage 37/38 and clearly detected thereafter (Fig. 5D). These results suggest that CaMKIx begins to appear at a relatively late stage of embryogenesis, and may play some physiological roles during these later stages.

Endogenous Substrates of CaMKIx and Upstream CaMKK—We found that CaMKIx phosphorylated various proteins obtained from animals other than *Xenopus* (Fig. 4 and Table 3). Since CaMKIx was found to be present abundantly in brain and heart, endogenous substrates for CaMKIx were investigated using extracts of these tissues. When the crude extract from heart was incubated with activated CaMKIx, a clear phosphorylated protein band of 26 kDa was observed (Fig. 6A, lane 8), suggesting that this protein might be one of the endogenous substrates of CaMKIx. The possibility that the 26-kDa protein is a truncated form of CaMKIx is unlikely, because this protein could not be immunostained by either a CaMKIx-specific antibody or an M8C antibody (data not shown). Although there were many phosphorylated proteins in brain extracts, these proteins were phosphorylated even in the absence of the activated CaMKIx (Fig. 6, lanes 2 and 4), suggesting that these protein phosphorylations are caused by various endogenous protein kinases such as CaMKI, CaMKII, and CaMKIV.

Although CaMKIx could be phosphorylated and activated by mouse recombinant CaMKKα (Figs. 3 and 4), a CaMKK in *Xenopus laevis* has not been reported so far. In order to clarify whether or not an upstream kinase of CaMKIx is present in the tissue extracts, the phosphorylation and activation of the enzyme were examined. When recombinant CaMKIx was incubated with extracts of brain, heart, and ovary, it was phosphorylated by the extracts of brain and heart (Fig. 7A, lanes 6 and 10), but not the ovary extract (Fig. 7A, lane 14). The phosphorylation of CaMKIx by the endogenous kinase in these extracts was solely dependent on Ca^{2+}/CaM (Fig. 7A, lanes 5, 6, 9, and 10).

Next, we examined whether or not the phosphorylation of CaMKIx by the endogenous protein kinase results in the activation of the enzyme. After CaMKIx was first phosphorylated by the endogenous protein kinase using cold ATP, the protein kinase activity of phosphorylated CaMKIx was examined by the addition of [γ-^{32}P]ATP and MBP. As shown in Fig. 7B, CaMKIx was activated by the addition of the heart, but not the ovary extract (lanes 10 and 14), suggesting that an upstream CaMKK is present in heart but not in ovary. Since the brain is rich in endogenous protein kinases such as CaMKII, CaMKIV, and CaMKI, MBP was phosphorylated even in the absence of

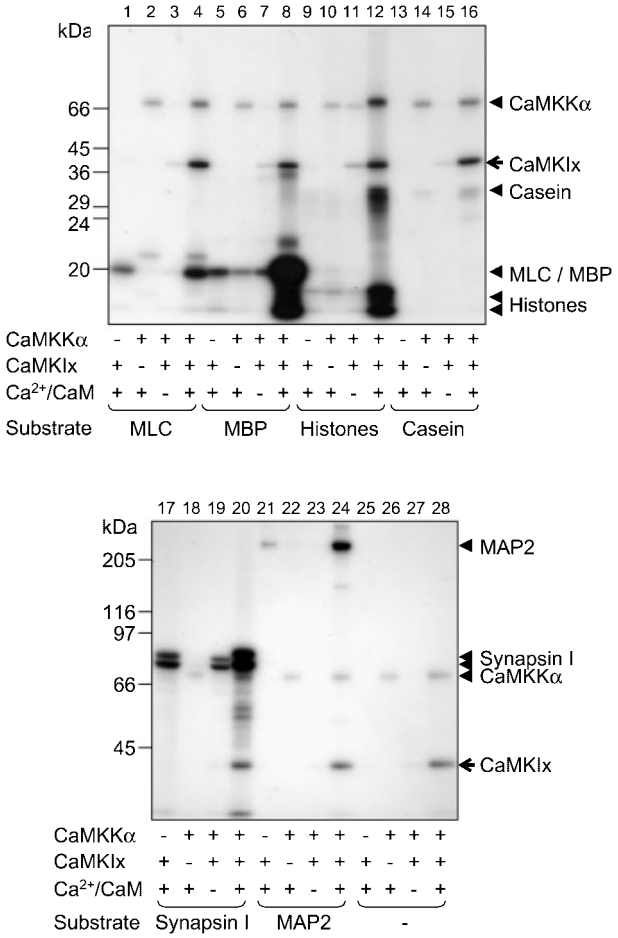

Fig. 4. **Phosphorylation of substrate proteins by CaMKIx.** As protein substrates, MLC (lanes 1–4), MBP (lanes 5–8), histones (lanes 9–12), casein (lanes 13–16), synapsin I (lanes 17–20), and MAP2 (lanes 21–24) were incubated under various conditions as indicated at the bottom of the autoradiogram. CaMKIx (100 ng), CaMKKα (50 ng), and Ca^{2+}/CaM were incubated in the presence (lanes 4, 8, 12, 16, 20, and 24) or absence (lane 28) of protein substrate in a final volume of 10 μl under the phosphorylation conditions described under "MATERIALS AND METHODS." Incubation was carried out with the same reaction mixture except that either CaMKKα (lanes 1, 5, 9, 13, 17, 21, and 25), CaMKIx (lanes 2, 6, 10, 14, 18, 22, and 26), or Ca^{2+}/CaM (lanes 3, 7, 11, 15, 19, 23, and 27) was omitted. After incubation at 30°C for 30 min, the reactions were stopped by the addition of 10 μl of SDS sample buffer, and 10-μl aliquots were resolved in a 12% polyacrylamide gel (lanes 1–16) or an 8% polyacrylamide gel (lanes 17–28). The phosphorylation of proteins was visualized by autoradiography. The migration positions of CaMKIx, CaMKKα, and the respective protein substrates are shown to the right of the autoradiograms.

Table 3. **Protein kinase activity of activated CaMKIx.** Phosphorylation of various protein substrates (0.1 mg/ml) or synthetic peptides (40 μM) by CaMKIx phosphorylated by CaMKKα was determined as described under "MATERIALS AND METHODS." The average values for specific activity were calculated from the results of four independent experiments.

Substrate	Ca^{2+}/CaM	Phosphorylation activity	
		nmol/min/mg	%
MBP	+	403	100
	–	29	7
MLC	+	38	9
	–	2	0
Histones	+	34	8
	–	2	0
Casein	+	0	0
	–	2	0
Synapsin I	+	224	56
	–	20	5
MAP2	+	28	7
	–	2	0
None	+	8	2
	–	0	0
Syntide-2	+	5644	100
	–	15	0
Kemptide	+	0	0
	–	0	0

exogenous CaMKIx under the experimental conditions used (Fig. 7B, lanes 3–6). However, MBP was phosphorylated more strongly when phosphorylated CaMKIx was added (Fig. 7B, lane 6), suggesting that CaMKK is also present in the *Xenopus* brain extract. These results, taken together, suggest that an upstream CaMKK is expressed in the tissues where CaMKIx is abundantly expressed.

CaMKIx Dephosphorylating Enzymes in Xenopus laevis—In the previous papers (*29*, *30*), we reported a unique protein phosphatase, designated as CaMKPase, that specifically dephosphorylates CaMKI, II, and IV, but not other protein substrates. In order to detect CaMKPase, we developed an in-gel protein phosphatase assay using a synthetic peptide corresponding to autophosphorylation site of CaMKII (*21*). In the present study, in order to examine whether or not CaMKPase is present in *Xenopus* tissue extracts, the in-gel protein phosphatase assay was carried out using ^{32}P-phosphorylated CaMKIx as a substrate. As shown in Fig. 8A, a protein phosphatase of 50 kDa was observed in brain (lane 2), heart (lane 3), and ovary (lane 4), but not in unfertilized eggs (lane 5). No 50-kDa enzyme activity was observed when ^{32}P-phosphorylated MBP was used as a substrate in gel (Fig. 8B), suggesting that this dephoshorylating activity is specific to CaMKIx. Since this enzyme migrates slightly faster than His_6-tagged rat CaMKPase (Fig. 8A, lane 1), the 50-kDa protein phosphatase was thought to be a homologue of *Xenopus* CaMKPase. Although two additional bands with molecular masses of 43 kDa and 30 kDa were also observed in the crude extract of heart (Fig. 8A, lane 3), the identities of these proteins are not known.

DISCUSSION

In the present study, cDNA clones for various protein kinases were isolated from cDNA libraries of *Xenopus* oocyte and *Xenopus* embryo at stage 28/30 by screening with kinase-specific monoclonal antibodies. Some protein kinases may be expressed throughout the embryogenesis of *Xenopus laevis*, while others may be expressed in a stage-specific manner. Most of the cDNA clones isolated were identified as well-known, previously reported Ser/Thr kinases (Tables 1 and 2). However, in addition to cDNA clones for well characterized protein kinases, one cDNA clone corresponding to a novel protein kinase that

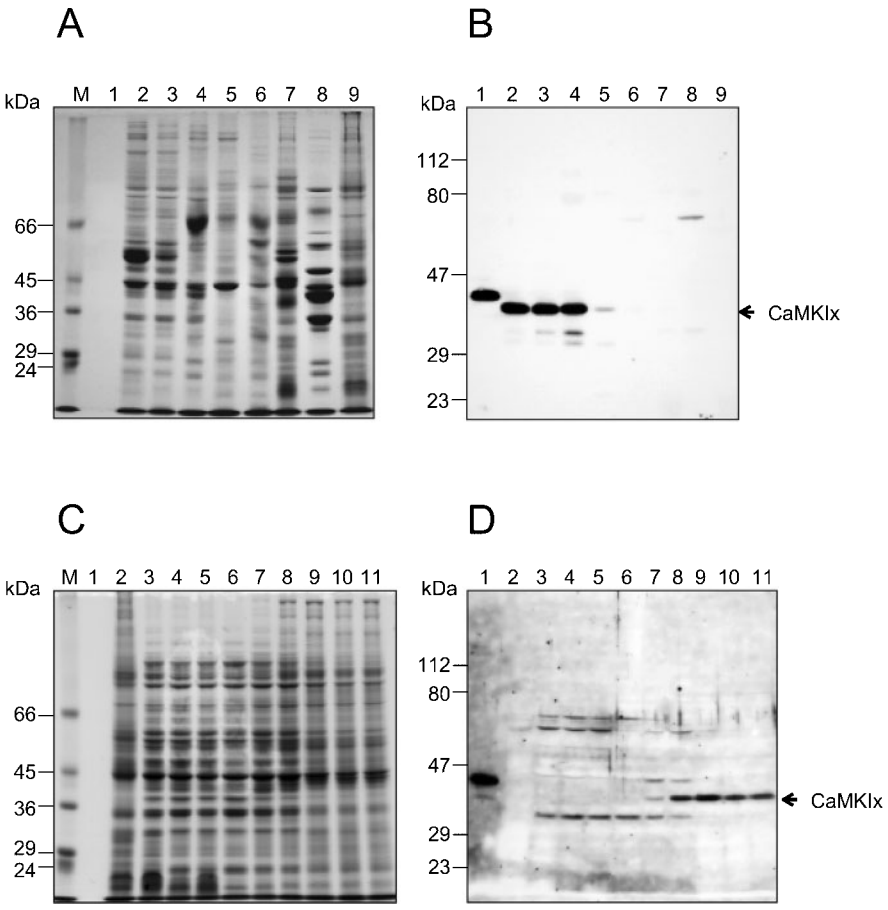

Fig. 5. **Expression of CaMKIx. A and B, tissue distribution of CaMKIx.** Crude extracts (10 μg protein) of *Xenopus* brain (lane 2), medulla oblongata (lane 3), heart (lane 4), spleen (lane 5), liver (lane 6), ovary (lane 7), skeletal muscle (lane 8), and unfertilized egg (lane 9), and recombinant CaMKIx (10 ng, lane 1) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (A). Alternatively, the resolved proteins were blotted onto a nitrocellulose membrane and CaMKIx was detected by Western blotting (B) as described under "MATERIALS AND METHODS." C and D, the expression of CaMKIx during embryogenesis of *Xenopus laevis*. Crude extracts (10 μg) of *Xenopus* unfertilized egg (lane 2), embryos at stage 7 (lane 3), stage 12 (lane 4), stage 20 (lane 5), stage 25/27 (lane 6), stage 37/38 (lane 7), stage 42 (lane 8), stage 45 (lane 9), stage 48 (lane 10), and stage 50 (lane 11), and recombinant CaMKIx (10 ng, lane 1) were applied to SDS-PAGE, and the proteins were stained with Coomassie Brilliant Blue (C). The resolved proteins were blotted onto a nitrocellulose membrane and analyzed by Western blotting (D). Lane M in (A) and (C) shows the molecular weight marker proteins. The migration position of CaMKIx is indicated by an arrow. Since recombinant CaMKIx is a His_6-tagged protein, it shows a slightly slower migration than that of the intact CaMKIx.

was not found on the database was isolated from the cDNA library of stage 28/30 embryos. Therefore, the cDNA clone for a novel protein kinase was further investigated in this study.

The predicted amino acid sequence of the putative kinase exhibited 75% identity to rat CaMKIβ2 (*24*) and, therefore, we designated it as CaMKIx. Very recently, while we were preparing this paper, Saneyoshi *et al*. reported two CaMKI isoforms, XCaM-KIα and XCaM-KI LiKβ, in *Xenopus laevis* (*31*). XCaM-KIα and XCaM-KI LiKβ show 64% and 59% identities to CaMKIx at the amino acid level, respectively, suggesting that these proteins are different isoforms of CaMKI in *Xenopus laevis*. Although the catalytic domains of these three enzymes are very similar, the amino-terminal and carboxyl-terminal sequences are all quite different.

In this study, we obtained a specific antibody to detect CaMKIx. This antibody clearly reacted with 38-kDa CaMKIx, and did not cross-react with protein bands of higher molecular masses (Fig. 5B), suggesting that XCaM-KIα (43 kDa) and XCaM-KI LiKβ (44 kDa) are not detected by the antibody. CaMKIx appeared to be abundantly expressed in adult *Xenopus* brain and heart as revealed by Western blotting (Fig. 5B). In contrast to CaMKIx, XCaM-KIα was also detected in muscle and oocytes, and CaM-KI LiKβ was detected in spleen and testis (*31*). When 10 μg of crude extract from brain or heart was analyzed by Western blotting using the specific antibody, 38-kDa CaMKIx was observed as an immunore-

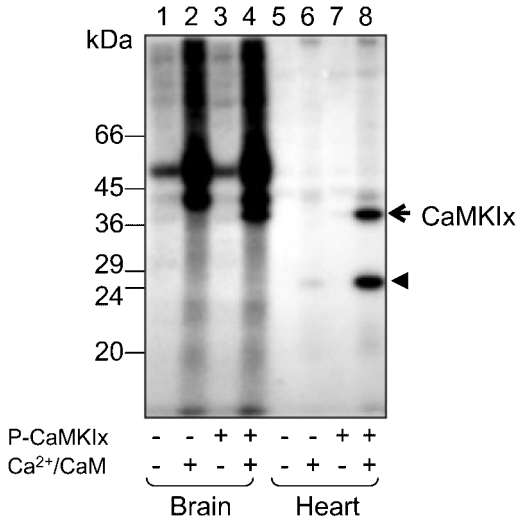

Fig. 6. **Endogenous substrates of CaMKIx.** Crude extracts (5 μg) of adult *Xenopus* brain (lanes 1–4) or heart (lanes 5–8) were incubated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) phosphorylated CaMKIx (40 ng) in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of Ca^{2+}/CaM in a final volume of 10 μl as described under "MATERIALS AND METHODS." After 20-min incubation at 30°C, the same volume of SDS sample buffer was added to the reaction mixture to terminate phosphorylation. Proteins were resolved by 12% SDS-PAGE and radioactive bands were detected by autoradiography. The migration positions of the molecular weight markers are shown on the left. The arrow and arrowhead indicate the positions of CaMKIx and the endogenous protein substrate in heart, respectively.

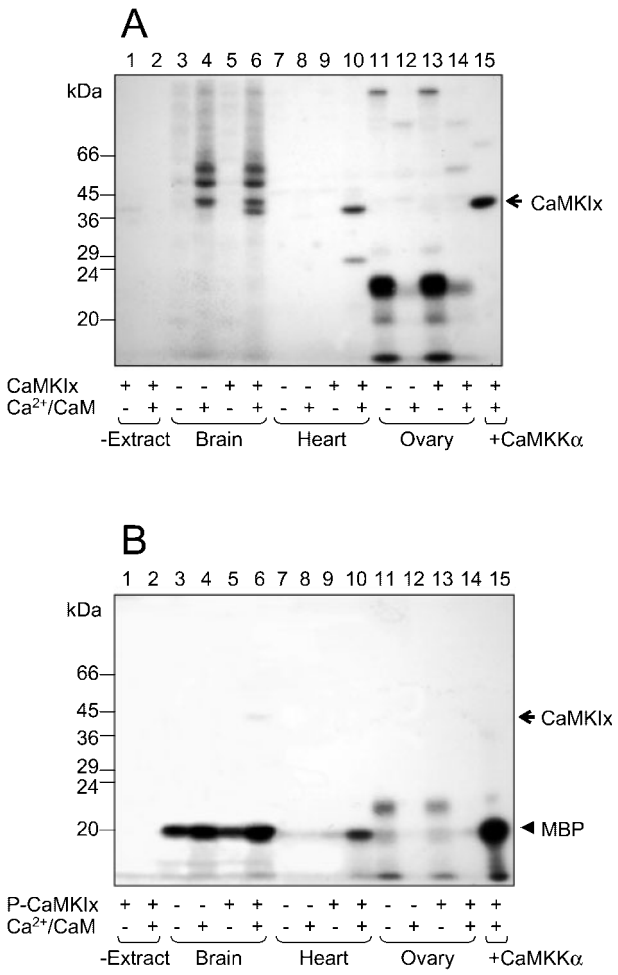

Fig. 7. **Phosphorylation and activation of CaMKIIx by an endogenous upstream protein kinase.** *A*, phosphorylation of CaMKIIx by a protein kinase in *Xenopus* tissue extracts. Recombinant CaMKIIx (0.5 μg) was incubated with crude extracts (5 μg) of brain (lanes 5 and 6), heart (lanes 9 and 10), and ovary (lanes 12 and 13), or with CaMKKα (10 ng, lane 15), or incubated without extract (lanes 1 and 2) in 10-μl reaction mixtures. The same experiments were carried out in the absence of CaMKIIx (lanes 3, 4, 7, 8, 11, and 12) as indicated at the bottom of the figure. Incubation was carried out in the presence (lanes 2, 4, 6, 8, 10, 12, 14, and 15) or absence (lanes 1, 3, 5, 7, 9, 11, and 13) of Ca^{2+}/CaM. After 20-min incubation at 30°C, the same volume of SDS sample buffer was added to the reaction mixture to stop phosphorylation. The protein samples were resolved by SDS-PAGE and analyzed by autoradiography. B, phosphorylation of MBP by phosphorylated CaMKIIx. Recombinant CaMKIIx was first incubated with crude extracts of brain (lanes 3–6), heart (lanes 7–10), and ovary (lanes 11–14), or CaMKKα (lane 15), or incubated without extract (lanes 1 and 2) as described in (A) except that cold ATP was used instead of [γ-^{32}P]ATP. MBP (1 μg) was incubated with pretreated CaMKIIx (20 ng) in the presence (lanes 2, 4, 6, 8, 10, 12, 14, and 15) or absence (lanes 1, 3, 5, 7, 9, 11, and 13) of Ca^{2+}/CaM at 30°C for 20 min in the standard phosphorylation mixture in a final volume of 10 μl containing 100 μM [γ-^{32}P]ATP. The reactions were stopped by the addition of 10 μl of SDS sample buffer and then 10-μl aliquots were applied to SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. The arrow and arrowhead indicate the positions of CaMKIIx and MBP, respectively. The migration positions of molecular weight markers are shown on the left.

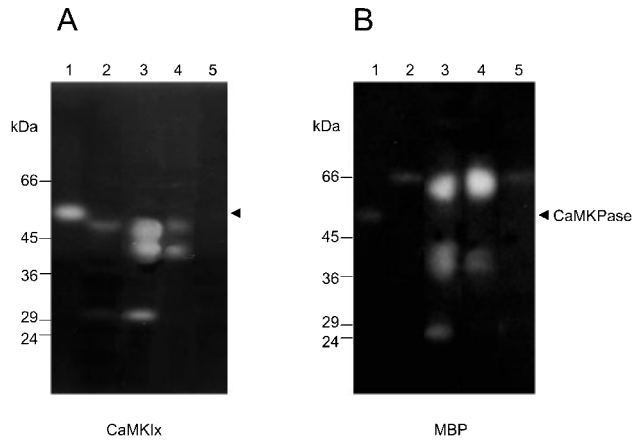

Fig. 8. **Detection of CaMKIIx dephosphorylating activities in *Xenopus* tissues by means of in-gel protein phosphatase assay.** Crude extracts (20 μg) of *Xenopus* brain (lane 2), heart (lane 3), ovary (lane 4), unfertilized egg (lane 5), and rat His_6-tagged CaMKPase (5 ng, lane 1) were electrophoresed in a 10% polyacrylamide gel containing 1×10^6 cpm [^{32}P]CaMKIIx (A) or [^{32}P]MBP (B) that had been phosphorylated previously. The resolved proteins were renatured *in situ*, and protein phosphatase activities were detected as described under "MATERIALS AND METHODS." The arrowhead indicates the position of His_6-tagged rat CaMKPase.

active band with a similar intensity to that observed with 10 ng of recombinant CaMKIIx. From these observations, the content of CaMKIIx was roughly estimated to be 0.1% of the total soluble proteins in brain or heart, indicating that CaMKIIx exists abundantly in these tissues.

In the present study, mouse recombinant CaMKKα was used to phosphorylate CaMKIIx. Although a CaMKK in *Xenopus laevis* has not been identified to date, the existence of such an upstream kinase was evident in the present study from the finding that CaMKIIx could be phosphorylated in the presence of crude brain and heart extracts, which contain abundant CaMKIIx, but not ovary extracts (Fig. 7B), in which CaMKIIx can not be detected (Fig. 5B). These results suggest that CaMKIIx is regulated by the upstream CaMKK homologue of *Xenopus laevis*.

The substrate specificity of CaMKIIx is somewhat different from that of previously reported CaMKI. CaMKI from rat brain phophorylates synapsin I as its most efficient substrate, and also phosphorylates histones, MLC, and MBP (*32*). On the other hand, CaMKIIx significantly phosphorylates MBP, and also synapsin I to a lesser extent (Table 3 and Fig. 4). However, both rat CaMKI and CaMKIIx phosphorylate various protein substrates, suggesting that these enzymes play important roles as multifunctional protein kinases. The physiological target(s) of CaMKI is one of the most important questions remaining to be solved. When endogenous substrates for activated CaMKIIx were examined using crude extracts of adult *Xenopus* tissues, a 26-kDa protein in the heart extract was clearly phosphorylated by the activated CaMKIIx in the presence of Ca^{2+}/CaM (Fig. 6). This 26-kDa protein was detected only in *Xenopus* heart, and not in other tissues or in a rat heart extract (data not shown), suggesting that this protein is a *Xenopus* heart-specific protein. The identity of this protein and its physiological function will be clarified in the near future.

The CaMKI cascade may be down-regulated by protein phosphatase(s) that dephosphorylate activated CaMKI. Using an in-gel protein phosphatase assay (*21*), three dephosphorylating activities were detected when ^{32}P-phosphorylated CaMKIx was used as an in-gel substrate (Fig. 8A). Although the identities of these proteins remain unclear, the 50-kDa enzyme appears to be *Xenopus* CaMKPase on the basis of its substrate specificity and mobility on SDS-PAGE. However, whether CaMKIx is down-regulated by the 50-kDa protein phosphatase or other protein phosphatases detected in this study remains unclear.

CaMKIx was isolated from a tadpole library but not from an oocyte library (Tables 1 and 2). Western blotting experiments also showed that CaMKIx is expressed at stages later than 25/27, suggesting that CaMKIx functions during the later stages of embryogenesis, especially in the central nervous system and heart. At least three CaMKI isoforms are evident in *Xenopus laevis*, CaMKIx, XCaM-KI LiKβ and XCaM-KIα. Although the tissue distribution and embryonic expression of XCaM-KI LiKβ and XCaM-KIα differ, the overexpression of these enzymes in HeLa cells produced essentially the same phenotype, suggesting that these CaMKIs are correlated with cell-structure regulation (*31*). However, since the timing of the expression of these three CaMKI isoforms is different, they may play different functional roles in the embryogenesis of *Xenopus laevis*. The question of how these isoforms share their functions remains to be elucidated.

This work was partly supported by a grant from the National Project on Protein Structural and Functional Analysis, and a grant from the Naito Foundation. This work was performed under the Cooperative Research Program of Institute for Protein Research, Osaka University. The nucleotide sequence data reported in this paper appear in the DDBJ with the accession number AB098710.

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