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Existence of Two Isoforms of Extracellular Signal-Regulated Kinase in Fish[1]

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Full-length cDNAs for extracellular signal-regulated kinases (ERK1 and ERK2) were isolated from a carp ovary cDNA library. The deduced amino acid sequences of carp ERK1 (cERK1) and ERK2 (cERK2) exhibited high degrees of homology to the known sequences of the ERK group. Northern blot analysis showed that cERK1 mRNA was not expressed in a tissue-specific manner, though the level of expression of cERK2 mRNA varied among tissues. Western blot analysis of the brain, kidney, and ovary confirmed the expression of cERK1 and cERK2 in carp. Our findings indicate that two isoforms of ERK, ERK1 and ERK2, exist in fish.

Key words: carp, extracellular signal-regulated kinase, fish, MAP kinase, mitogen-activated protein kinase.

Extracellular signal-regulated kinases, ERK1 and ERK2, are the key enzymes in the intracellular signal transduction pathway (*1-4*). They are also termed mitogen-activated protein (MAP) kinases because they were purified on the basis of their activation in response to growth stimulation by mitogens (*5, 6*). ERKs are known to participate in the last step of the MAP kinase cascade, being activated through phosphorylation at Thr (T) and Tyr (Y) in a TEY tripeptide motif by the upstream MAP kinase kinases (*1-4*).

Recent studies have shown that mammalian cells contain multiple isoforms of ERK having a TEY motif: ERK1, ERK2, and ERK5 have been identified in man (*7-10*), and ERK1 and ERK2 in rat (*5, 11, 12*). On the other hand, only one type of ERK has been reported in lower animals: *Xenopus* (MPK1) (*13, 14*) and *Drosophila* (DmERK-A) (*15*). In yeast, however, there are three MAP kinases, MPK1/SLT2 (*16, 17*), FUS3 (*18*), and KSS1 (*19*), possessing the common TEY motif. Therefore, the existence of multiple isoforms of ERK in lower animals remains unclear. In this paper, we report the existence of two ERK isoforms (cERK1 and cERK2) in lower vertebrates, fish.

MATERIALS AND METHODS

Preparation of RNA—Total RNAs from various tissues of a female carp (*Cyprinus carpio* L.) were isolated by ultracentrifugation in 5.7 M cesium chloride after homogenization in 4 M guanidinium thiocyanate (*20*). Poly(A)$^+$ RNA was enriched by chromatography on oligo (dT) cellulose (Collaborative Biomedical Products) or on Oligotex-dT30 (Takara).

RT-PCR Cloning—To obtain a partial sequence of carp ERK, PCR was conducted with degenerate oligonucleotide primers having the following sequences: 5′-GGNGARGG-NGCNTAYGG-3′ as the sense primer and 5′-ARRTCRTC-NARYTCCAT-3′ as the antisense primer for the first PCR, and 5′-NTTYGARCAYCARAC-3′ as the sense primer and 5′-GGRTCRTARTAYTGYTC-3′ as the antisense primer for the second PCR, where N=A+G+C+T, R=A+G, and Y=C+T. These primers correspond to the amino acid sequences, GEGAYG, MELDDL, PFEHQT, and EQYYDP, respectively, which are identical in human ERK1 (*9*) and ERK2 (*7*), rat ERK1 (*5*), and ERK2 (*11*), and *Xenopus* MPK1 (*14*) (shown in Fig. 1). Single-stranded cDNA was synthesized from 1 μg of total RNA of ovary with 200 units of MMLV-RT (Gibco-BRL) in a 20 μl reaction mixture comprising 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM each dNTP, 5 μM random hexamer, and 20 units of ribonuclease inhibitor RNasin (Toyobo). A quarter of the resultant cDNA was used as a template for the first PCR, which was carried out in a 25 μl solution comprising 8 units of ΔTth DNA polymerase (Toyobo), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 mM each dNTP, and 0.2 μM each primer. The second PCR was performed

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Abbreviations: bp, base pair; cDNA, complementary DNA; dNTP, deoxynucleotide triphosphate; ERK, extracellular signal-regulated kinase; kb, kilobase; MAP kinase, mitogen-activated protein kinase; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard sodium chloride/sodium citrate buffer.

using 1 μl of the first PCR reaction mixture in a 50 μl solution comprising 2.5 units of ΔTth DNA polymerase (Toyobo), 0.5 μM each primer, and 0.2 mM each dNTP. The conditions for both PCRs were 30 s at 95°C, 1 min at 56°C, and 2 min at 72°C for 25 cycles (Astec, Program Temp Control System PC-700). The resultant products of the expected size, approximately 600 bp, were subcloned in pBluescript II KS$^-$ (Stratagene). DNA sequencing analysis revealed that there were two distinct cDNA fragments in the pool of PCR products.

cDNA Cloning and Sequencing—The synthesis of double-stranded cDNA was accomplished with a ZAP-cDNA Synthesis Kit (Stratagene) using poly(A)$^+$ RNA prepared from ovary by chromatography on oligo (dT) cellulose. The cDNAs were size-fractionated on a CHROMA SPIN-100 column (Clontech) and then adapter-ligated prior to the generation of an oligo (dT) primed library in Uni-ZAP/*Eco*RI/*Xho*I/CIAP (Stratagene).

The cDNA library was screened with a mixture of the two distinct cDNA fragments obtained on PCR as probes. Plaque hybridization was performed at 42°C in a solution comprising 5×SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS, 50% formamide, 100 μg/ml denatured salmon sperm DNA, 0.6% Ficoll 400, 0.6% polyvinylpyrrolidone 100, 0.6% BSA, and ^{32}P-labeled probe prepared with a Megaprime DNA labeling system (Amersham International plc). After hybridization, the filters were washed four times with 2×SSC at room temperature and then three times with 2×SSC, 0.5% SDS at 65°C prior to autoradiography. Plasmids (pBluescript SK$^-$) containing the cDNA insert were obtained by *in vivo* excision from positive single plaques according to the manufacturer's instructions (Stratagene). After subcloning into pBluescript II KS$^-$, DNA fragments were sequenced by a PCR procedure involving fluorescent dideoxynucleotides and a model 373A automated sequencer (Applied Biosystems). The sequences reported in this paper were determined for both strands.

Southern and Northern Blot Analyses—A genomic Southern experiment was performed by means of the standard procedure (*20*) using 10 μg of carp genomic DNA digested with restriction enzymes (Toyobo). Northern analysis was carried out using poly(A)$^+$ RNA (1 μg) prepared from various tissues by chromatography on Oligotex-dT30. In both analyses, the probes used for hybridization were prepared from the two distinct cDNA fragments isolated by RT-PCR cloning. Hybridization was performed under the same conditions as described above.

Western Blot Analysis—Tissue homogenates were prepared from the brain, kidneys, and ovaries of a carp in Laemmli sample buffer, at a concentration of 40 mg tissue/ml. Nine microliters of each sample was resolved by 10% SDS-PAGE, followed by transfer to a PVDF filter (Atto). After blocking in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 and 5% skimmed milk, the filter was incubated with anti-MAP kinase antibodies (*21*) for 1.5 h, and then washed in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20. A goat anti-rabbit IgG conjugated to alkaline phosphatase (Santa Cruz Biotechnology) was used as the secondary antibody. Immunoreactive bands were detected in a solution comprising 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 7.8 mM nitroblue tetrazolium, and 4 mM 5-bromo-4-chloro-3-indolyl phosphate. Alternatively, they were detected by means of the ECL detection system (Amersham). The anti-MAP kinase polyclonal antibodies (αMAP kinase-301) were raised against residues 301-323 (RIEVEQALAHPYLEQYYDPS-DEP) of human ERK2 (*7, 8*), and recognize both ERK1 and ERK2 (*21*). This peptide sequence is found at residues 332-354 of cERK1 and residues 310-332 of cERK2, although two residues, Glu (E)-303 and Gln (Q)-306, are replaced by Thr (T) and Glu (E) in cERK1, and two, Gln (Q)-306 and Ser (S)-320, by Glu (E) and Thr (T) in cERK2, respectively. The anti-MAP kinase monoclonal antibodies (αMAP kinase-325) which were raised against a synthetic peptide corresponding to positions 325-345 (LAHPYLEQYYDPT-DEPVAEEP) of human ERK1 were purchased from Transduction Laboratories (Catalog No. 12320).

DNA Constructions and Preparation of GST Fusion Proteins—The cDNA fragments encoding the ORF of cERK1 and cERK2 were amplified by PCR using pairs of primers, 5′-CGGGATCCATGGCGGAATCGGGCAGCAG-3′ (sense) and 5′-AAGAATTCTCAGGAGCCCTGGTAAG-TGG-3′ (antisense) for cERK1, and 5′-CGGAATTCATAT-GGCGACAGCTGCGGTATC-3′ (sense) and 5′-CGGAAT-TCCTATGGTCTGTAGCCTGGCT-3′ (antisense) for cERK2, respectively. To yield pGEX-2T-cERK1 and pGEX-2T-cERK2, the ORF cDNAs were cloned at the *Bam*HI/*Eco*RI sites or *Eco*RI/*Eco*RI sites of pGEX-2T vector (Pharmacia). GST fusion proteins (GST-cERK1 and GST-cERK2) were bacterially expressed and purified following to the manufacturer's instructions. Purified proteins (5 μg) were treated with thrombin (Sigma, 0.5 unit/ml) at 37°C for 30 min in a 20 μl reaction buffer comprising 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2.5 mM $CaCl_2$.

RESULTS AND DISCUSSION

The RT-PCR strategy for the cloning of partial cDNA of carp ERK successfully yielded two distinct cDNA fragments of approximately 600 bp. DNA sequencing analysis revealed that one of the two fragments was rather similar to rat ERK1 and the other to rat ERK2 (data not shown).

In order to obtain full length cDNAs for carp ERK1 and ERK2, a carp ovary cDNA library was screened with a mixture of the two PCR-derived cDNA fragments as probes. On the screening of 10^5 independent recombinant phages, five positive clones were isolated. The restriction maps indicated that they could be divided into two types (data not shown). As a result of determination of the nucleotide sequence of the longest clone of each type, two of the five clones were revealed to encode a carp ERK1 homolog (cERK1, accession number AB006038) and the others a carp ERK2 homolog (cERK2, accession number AB006039). The cDNAs of cERK1 and cERK2 consist of 1,539 and 1,658 nucleotides with poly(A) tails, respectively. The in-frame stop codons in the 5′ and 3′ regions of both cDNAs indicated that these clones contain the entire cERK1 and cERK2 coding regions. The coding region for cERK1 comprises 1,176 nucleotides and corresponds to 392 amino acids with a predicted mass of 44.1 kDa. The coding region for cERK2 comprises 1,107 nucleotides and corresponds to 369 amino acids with a predicted mass of 42.2 kDa. Both cERK1 and cERK2 contain, in the dual phosphorylation site, a TEY tripeptide motif, which is a common

feature of the ERK group in the MAP kinase superfamily (*1-4*). Comparison with protein sequences in the SWISS-PROT database by means of FASTA indicated that cERK1 is 86.5, 86.9, 86.3, 86.6, 85.0, 79.1, 49.7, 52.2, and 50.8%, and cERK2 is 83.1, 92.9, 83.1, 94.9, 94.4, 78.7, 49.6, 51.3, and 51.4% identical in primary structure to human ERK1 (*9*) and ERK2 (*7*), rat ERK1 (*5*) and ERK2 (*11*), *Xenopus* MPK1 (*14*), *Drosophila* DmERK-A, and yeast MPK1/SLT2 (*16, 17*), FUS3 and KSS1 (*18, 19*), respectively (Fig. 1). Although cERK1 shows apparently higher homologies to ERK2s than ERK1s, we referred to the ERK with the larger molecular weight as cERK1 (Fig. 1).

Figure 2 shows the results of Southern blot analysis, which was performed with carp genomic DNA. The hybridization pattern obtained with the cERK1 or cERK2 RT-PCR fragment indicates that these ERKs are encoded by different genes. In other words, the carp genome has two distinct genes for cERK1 and cERK2.

Using each of the RT-PCR fragments of cERK1 and cERK2 as a probe, Northern blot analysis was conducted

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cERK1     carp          1  MAESGSSAAA AGAAGSSSSA AGAGAAVAVG GANGAAGPKP GLESVKGQNF DVGPRYTDLQ
cERK2     carp          1                        MAT.AVSA P.G.GPN.GS .A.M.R..A. .......N.S
ERK1      rat           1                MAAAAAA P.G.GGEPR. T.GVVPVVPG EV.V....P. .......Q..
ERK2      rat           1                                    MAAAAAA .P.M.R..V. .......N.S
MPK1      Xenopus       1                                 MA A.AASSN.GG .P.M.R..A. .......N.S
DmERK-A   Drosophila    1                          ME EFNSSGSV.N .TGSTEV.QS NA.VIR..I. E.....IK.A
FUS3      yeast         1                                                    MP.RIVY NISSDFQLKS
KSS1      yeast         1                                                    MARTIT. .IPSQ.KLVD

cERK1     carp         61  YI[redacted]MV CSAFDNVNKI RVAIKKIS[redacted]YCQRTL REIKILLRFR -HENIIGIND
cERK2     carp         39  .......... ...YKRD..V .......... .......... .........K -.........
ERK1      rat          48  .......... S..Y.H.R.T .......... .......... ...Q...G.. -...V...R.
ERK2      rat          28  .......... ...Y..L..V .......... .......... .......... -.........
MPK1      Xenopus      33  .......... ...HC.I..V .......... .......... .........K -.........
DmERK-A   Drosophila   43  .......... V..D.TLTNQ .......... .......... ...T..T..K -.....D.R.
FUS3      yeast        18  LL......V. ...THKPTGE I......E.. DKPLFAL... ......KH.K -.....T.FN
KSS1      yeast        18  L.......T. ...IHKPSG. K......Q.. SKKLFVT..I ....L.RY.H E.....S.L.

cERK1     carp        120  ILRARRIEYM RDVYIVQDLM ETDLYKLLKT QQ-----LSN DHVCYFLYQI LRGLKYIHSA
cERK2     carp         98  .I.TTT.DQ. K......... .......... .H-----... ..I..... ..........
ERK1      rat         107  .....TL.A. .......... .........S ..-----... ..I..... ..........
ERK2      rat          87  .I..PT..Q. K......... .......... .H-----... ..I..... ..........
MPK1      Xenopus      92  .I..PT..Q. K......... .......... .H-----... ..I..... ..........
DmERK-A   Drosophila  102  ...VDS.DQ. .......C.. .......... .R-----... ..I..... ..........
FUS3      yeast        77  .Q.TDSF.NF NE...I.E.. Q...HRVIS   M-----..D ..IQ..I..T ..AV.VL.GS
KSS1      yeast        78  KV.PVS.DKL NA..L.EE.. ....Q.VINN .NSGFST..D ...Q..T... ..A..S....

cERK1     carp        175  NVLHRDLKPS NLLINTTCDL KICDFGLARI -----AD--- PEHDHTGFLT EYVATRWYRA
cERK2     carp        153  .......... ...L...... .........V -----..--- .D........ ..........
ERK1      rat         162  .......... .......... .......... -----..--- .......... ..........
ERK2      rat         142  .......... ...L...... .........V -----..--- .D........ ..........
MPK1      Xenopus     147  .......... ...L...... .........V -----..--- .D........ ..........
DmERK-A   Drosophila  157  .......... .....K.... .......... -----..--- .......... ..........
FUS3      yeast       132  ..I....... .....SN... .V........ IDESA..NSE .TGQQS.-M. ..........
KSS1      yeast       138  Q.I...I... ...L.SN... .V.......C L----.SSSD SRETLV..M. ..........

cERK1     carp        227  PEIMLNSKGY TKSIDMWSVG CILAEMLSNR PIFPGKHYLD QLNHILGILG SP-TQDDLNC
cERK2     carp        205  .......... .....I.... .......... .......... ..-S.E....
ERK1      rat         214  .......... .....I.... .......... .......... ..-S.E....
ERK2      rat         194  .......... .....I.... .......... .......... ..-S.E....
MPK1      Xenopus     199  .......... .....I.... .......... .......... ..-S.E....
DmERK-A   Drosophila  209  .......... .....I.... .......... .......V.. ..-SR...E.
FUS3      yeast       191  ..V..T.AK. SRAM.V..C. .....LFLR. .....RD.RH ..LL.F..I. T.HSDN..R.
KSS1      yeast       194  .....TFQE. .TAM.I..C. ......V.GK .L...RD.HH ..WL..EV.. T.-SFE.F.Q

cERK1     carp        286  IINMKARNYL QALPQKPKIP WNKLFPKAD- -NKALDLLDR MLTFNPIKRI TVEEALAHPY
cERK2     carp        264  ...I...... LS..LRC.V. ..R...N..- -P.......K ......H... E.........
ERK1      rat         273  .......... .S..S.T.VA .A.....S.- -S........ ......N... ..........
ERK2      rat         253  ...L...... LS..H.N.V. ..R...N..- -S.......K ......H... E..Q......
MPK1      Xenopus     258  ...L...... LS..H.N.V. ..R...N..- -P.......K ......H... E..A......
DmERK-A   Drosophila  268  ...E...... ES..F..NV. .A....N..- -AL.....GK ......H... P.........
FUS3      yeast       251  .ESPR..E.I KS..MY.AA. LE.M..R--V NP.GI...Q. ..V.D.A... TAK...E...
KSS1      yeast       253  .KSKR.KE.I AN..MR.PL. .ETVWS.T.L NPDMI....K ..Q...D... SAA...R...

cERK1     carp        344  L[redacted]SDE PVAE----EP FTFN[redacted] PKEKLKELIY EETARFQATY QGS*
cERK2     carp        322  .......T.. ....----A. .K.D...... ...T.....F .......PG. RP*
ERK1      rat         331  .......T.. ....----.. ...D...... ...R.....F Q......PGA PEAP*
ERK2      rat         311  .......... .I..----A. .K.D...... .........F .......PG. RS*
MPK1      Xenopus     316  .......... ....----A. LK.E...... ...T.....F .......PG. *
DmERK-A   Drosophila  326  .......G.. ....----V. .RI...N..I SRDA..S..F ...LK.KERQ PDNAP*
FUS3      yeast       309  .QT.H..N.. .EG.PIPPSF .E.DHYKEA. TTKD..K..W N.IFS*
KSS1      yeast       313  .AM.H..... .E---YP--. LNLDD.FWK. DNKIMRPEEE ..VPIEMLKD MLYDELMKTM

KSS1      yeast       368  E*
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Fig. 1. **Comparison of the amino acid sequences of cERK1 and cERK2 with those of rat, *Xenopus*, *Drosophila*, and yeast MAP kinases.** The sequences comprise our results, and those for rat ERK1 (*5*) and ERK2 (*11*), *Xenopus* MPK1 (*14*), *Drosophila* DmERK-A (*15*), and yeast FUS3 (*18*) and KSS1 (*19*). Identical residues are denoted by periods, and gaps introduced into the sequences are depicted by hyphens. The light shadowed sequence denotes the TEY motif. The dark shadowed sequences represent the amino acid sequences from which the degenerate PCR primers were designed.

(Fig. 3). cERK1 mRNA of approximately 5 kb was evident in every tissue examined. Additional signals of 2.4 and 1.9 kb mRNAs were detected most strongly in ovary. On the other hand, cERK2 mRNA of approximately 5 kb was expressed in most tissues but at different levels, being undetectable in liver and ovary, though a strong signal of 2 kb mRNA was observed in ovary. cERK2 mRNA was expressed strongly in brain and kidney, while cERK1 mRNA was abundant ubiquitously. These results suggest that the expression of cERK1 and cERK2 is regulated differently at the transcriptional level. These kinases might have distinct functions in tissues.

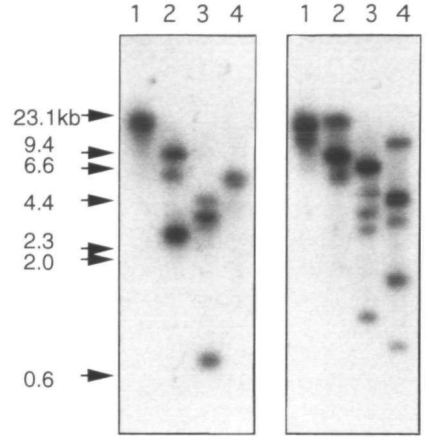

Fig. 2. **Southern blot analysis of carp genomic DNA.** Carp genomic DNA (10 μg) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), or *Pst*I (lane 4), separated by electrophoresis on a 0.8% agarose gel, and then transferred to a nylon membrane. The blot was then hybridized to ^{32}P-labeled PCR-derived cERK1 (left) and cERK2 (right) fragments. The membrane was washed at 65°C in 2× SSC containing 0.5% SDS prior to autoradiography. The positions of DNA size markers and their lengths in kilobases are indicated.

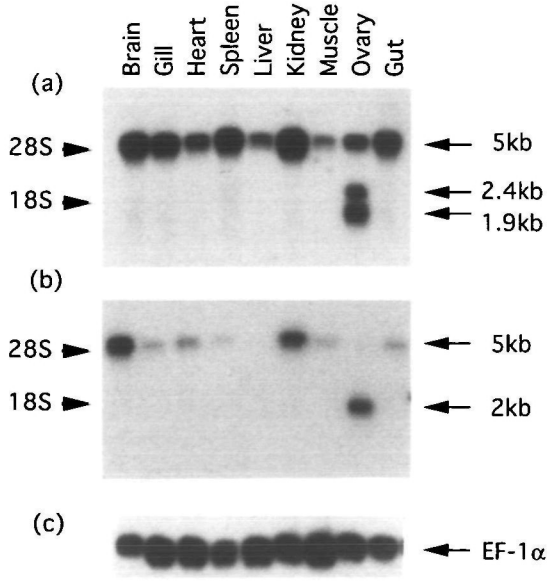

Fig. 3. **Tissue distributions of cERK1 and cERK2 mRNAs.** Poly(A)$^+$ RNA samples were prepared from brain, gill, heart, spleen, liver, kidney, muscle, ovary, and gut. Each RNA sample (1 μg) was separated by 1% agarose-formaldehyde gel electrophoresis, transferred to a nylon membrane, and then fixed. ^{32}P-labeled PCR-derived cERK1 (a) and cERK2 (b) fragments were used as probes. After hybridization, the membranes were washed at 65°C in 2×SSC containing 0.5% SDS. The positions of 28S and 18S rRNA are indicated on the left. The blot was reprobed with medaka elongation factor-1α (EF-1α) cDNA as an internal control (c).

To confirm the existence of the two ERKs, cERK1 and cERK2, at the protein level, Western blot analysis was performed using anti-MAP kinase antibodies (*21*) to tissue homogenates of kidney and brain, in which cERK and cERK2 mRNAs, respectively, were expressed most abundantly. Ovary was also examined because the cDNAs of cERK1 and cERK2 had been isolated from an ovary cDNA library. As a result, immunoreactive bands corresponding to approximately 44 and 42 kDa were detected in these tissues (Fig. 4a, αMAP kinase-301). Although the sequence of the peptide used as an antigen to raise the antibodies used here differs by two residues from the corresponding sequence in cERK1 or cERK2, the sizes of the detected proteins were consistent with the expected molecular weights of cERK1 and cERK2 (Fig. 4, a and b). Upon SDS-PAGE, cERK1 and cERK2 migrated closer to each other than ERK1 and ERK2 of mouse (BALB/3T3). When a commercially obtained anti-MAP kinase monoclonal antibody was used, only a 42 kDa protein was detected (Fig. 4, a and b, αMAP kinase-325). This result rules out the possibilities that the 42 kDa immunoreactive band shown in Fig. 4a was a degraded product of the 44 kDa protein and that the 44 kDa band was a phosphorylated form of the 42 kDa protein. Therefore, these results imply that the cERK1 and cERK2 proteins are actually expressed in the carp tissues.

The findings in this study indicate that at least two

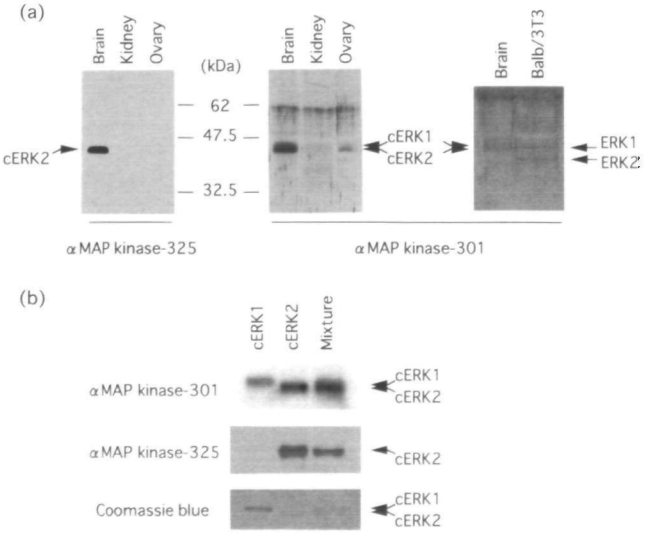

Fig. 4. **Detection of the cERK1 and cERK2 proteins in tissues.** (a) Samples were prepared from brain, kidney and ovary. Both of cERK1 and cERK2 were detected with αMAP kinase-301, whereas only cERK2 was evident with αMAP kinase-325. The positions of immunoreactive bands in brain were compared with those of BALB/3T3 (αMAP kinase-301). The positions of molecular size markers (kDa) are indicated. (b) Recombinant cERK1 and cERK2 proteins were obtained by digestion of GST-cERK1 and GST-cERK2 with thrombin. They were subjected to 10% SDS-PAGE followed by Coomassie Blue-staining or Western blotting.

isoforms of ERK exist in fish. This is very interesting from the viewpoint of molecular evolution, because only one type of ERK has been identified in *Xenopus* (MPK1) (*13, 14*) and *Drosophila* (DmERK-A) (*15*). However, further study is needed to clarify if any other ERK-related kinases corresponding to human $p63^{mapk}$ (*8*) and ERK5 (*10*) exist.

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