Calmodulin of Olive Flounder *Paralichthys olivaceus* : Cloning and Expression Analysis

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Abstract Calmodulin (CaM) is a Ca2+-binding protein essential for biological functions mediated through Ca2+-dependent mechanism. A cDNA clone for CaM was isolated from a cDNA library of olive flounder *Paralichthys olivaceus*. The CaM cDNA concists of 782 bp and encodes a polypeptide of 149 amino acids with four Ca2+-binding motifs EF-hands (EF-I, EF-II, EF-III, and EF-IV). The deduced amino acid sequence of CaM shows 97-100% amino acid sequence identity to other CaM sequences. Semi-quantitative PCR analysis revealed that the CaM transcription was began during early development and the CaM mRNA is expressed highly in brain and intestine, and moderately in kidney, gill, and eye of healthy olive flounder. Taken together, CaM may be necessary for early olive flounder development and that it may have a part in homeostasis.

Key words : Calmodulin, Ca2+-binding motif, olive flounder, *Paralichthys olivaceus*

Introduction

Calmodulin (CaM), a Ca2+-binding protein expressed in all eukaryotic cells, serves as a major intracelluar Ca^{2+} sensor and signal transducer in living cells. The amino acid sequence of calmodulins has been highly conserved from protozoans through vertebrates [9,10, 16,19]. CaM binds to and regulates a number of different protein targets, thereby affecting many different cellular functions. CaM mediates processes such as inflammation, metabolism, apoptosis, muscle contraction, intracellular movement, short-term and long-term memory, nerve growth and host defense [2,4,5,12,18,21]. Line of evidence also demonstrates that CaM plays an important role in regulating the function of mature osteoalsts and osteoalsogenesis, a bone bimeneralization related process [22].

CaM is expressed in many cell types and has different subcellular locations, including the cytoplasm, within organelles, or associated with the plasma or organelle membranes. CaM also makes use of the cal-

cium stores in the endoplasmic reticulum, and the sarcoplasmic reticulum. The molecular structure of CaM is characterized by the presence of four Ca^{2+}-binding motifs known as the helix-loop-helix EF-hands [20]. Upon Ca^{2+} binding, CaM undergoes a conformational change upon binding to calcium, which enables it to bind to Ca^{2+}-sensitive target proteins for a specific response [13].

From non-mammalian vertebrates, two CaM cDNAs were isolated from frog *Xenopus laevis* and four distinct CaM-encoding DNAs were cloned from medaka *Oryzias latipes* [3,11]. Six CaM genes coding for a 100% identical CaM protein molecule present in the zebrafish genome (alpha, alpha2, beta, beta2, gamma and gamma 2) [6]. Moreover, several EST clones from olive flounder *Paralichthys olivaceus* leukocyte cDNA library showed high identity to human CaM, but those contain a part of the homologous region. In the present study, we isolated and characterized the full-length cDNA of CaM from olive flounder *P. olivaceus*.

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Materials and Methods

Sequencing and sequence analysis

A clone from the cDNA library of olive flounder was isolated using Plasmid Miniprep Kit (Qiagen), and was thoroughly sequenced using both the T7 forward and SP6 reverse primers (Promega) in Automatic sequencer ABI3730xl (Applied Biosystems Inc.). Analysis of potential open reading frame (ORFs) and comparison of amino acid sequences (or nucleotide sequences) were performed with the ORF finder and BLAST programs on National Center for Biotechnology Information website.

Multiple alignmenst

The multiple sequence alignment was created by using the CLUSTAL W [8], and the same software was used to analyze similarity of the aligned sequences using a neighbor joining (NJ) algorithm. Putative phosphorylation sites were predicted by NetPhos 2.0 Server with high scores [1].

RNA extraction

Total RNA was extracted from tissues of healthy olive flounder using TRIzol reagent (Invitrogen) using a homogenizer. First-strand cDNA was synthesized using the Advantage RT-for-PCR kit (BD Biosciences). The total RNA was quantitatively determined to the same concentration, and a sample of total RNA at 1 μg was used for reverse transcription.

Semi-quantitative RT-PCR analysis

The first strand cDNA was synthesized using the Advantage RT-for-PCR kit (BD sciences). The gene specific primers CaM-F (5′-TGATGACAGCGAAGTG AAGG-3′) and CaM-R (5′-TCCACGAGCATCTGGTT GTA-3′) were used to amplify the CaM transcript. The primers GAPDH-F (5′-TCCCATGTTCGTCATGGGC GTGA-3′) and GAPDH-R (5′-ATTGAGCTCAGGGAT GACCTTG-3′) were used to amplify the GAPDH fragment as an internal control. CaM cDNAs were amplified by PCR under the following conditions: denaturing at 94°C for 5 min followed by 30 cycles of amplification at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec in a thermal cycler. For GAPDH: denaturing at 94℃ for 5 min followed by 25 cycles of at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec. The

PCR products were examined by electrophoresis on a 1.5% agarose gel.

Results and Discussion

Cloning analysis of olive flounder calmodulin (CaM) cDNA

Previously, we constructed a cDNA library of the olive flounder *P. olivaceus* and carried out expressed sequence tag (EST) analysis (data not shown). We isolated the one clone, FLDS-2-B9, whose deduced amino acid sequence showed very high homology to those of CaM proteins from various biological sources. The full-length *Calmodulin (CaM)* cDNA is 782 bp long and consists of a 5' UTR of 81bp, an open reading frame of 450 bp that encoding a 149-amino acid protein and a 3' UTR of 251 bp (GenBank accession number EU519228) (Fig. 1).

Sequence analysis of olive flounder CaM cDNA

The deduced amino acid sequence of *CaM* cDNA shared 100% amino acid identity with the those of *Rattus norvegicus* (GenBank accession no. NP_1 14175), *Homo sapiens* (NP_008819), *Danio rerio* (NP_998516) and *Xenopus laevis* (NP_001080864). It also shared 97 and 99% amino acid identity with those of *Strongylocentrotus purpuratus* (XP_780862) and *Pan troglodytes* (BAF62378), respectively.
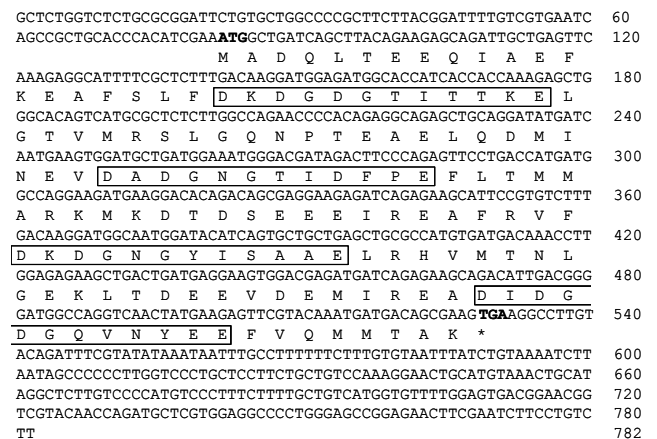
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GCTCTGGTCTCTGCGCGGATTCTGTGCTGGCCCCGCTTCTTACGGATTTTGTCGTGAATC  60
AGCCGCTGCACCCACATCGAAATGGCTGATCAGCTTACAGAAGAGCAGATTGCTGAGTTC  120
                      M  A  D  Q  L  T  E  E  Q  I  A  E  F
AAAGAGGCATTTTCGCTCTTTGACAAGGATGGAGATGGCACCATCACCACCAAAGAGCTG  180
K  E  A  F  S  L  F  D  K  D  G  D  G  T  I  T  T  K  E  L
GGGCACAGTCATGCGCTCTCTTGGCCAGAACCCCACAGAGGCAGAGCTGCAGGATATGATC  240
G  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I
AATGAAGTGGATGCTGATGGAAATGGGACGATAGACTTCCCAGAGTTCCTGACCATGATG  300
N  E  V  D  A  D  G  N  G  T  I  D  F  P  E  F  L  T  M  M
GCCAGGAAGATGAAGGACACAGACAGCGAGGAAGAGATCAGAGAAGCATTCCGTGTCTTT  360
A  R  K  M  K  D  T  D  S  E  E  E  I  R  E  A  F  R  V  F
GACAAGGATGGCAATGGATACATCAGTGCTGCTGAGCTGCGCCATGTGATGACAAACCTT  420
D  K  D  G  N  G  Y  I  S  A  A  E  L  R  H  V  M  T  N  L
GGAGAGAAGCTGACTGATGAGGAAGTGGACGAGATGATCAGAGAAGCAGACATTGACGGG  480
G  E  K  L  T  D  E  E  V  D  E  M  I  R  E  A  D  I  D  G
GATGGCCAGGTCAACTATGAAGAGTTCGTACAAATGATGACAGCGAAGTGAAGGCCTTGT  540
D  G  Q  V  N  Y  E  E  F  V  Q  M  M  T  A  K  *
ACAGATTTCGTATATAAATAATTTGCCTTTTTCTTTGTGTAATTTATCTGTAAAATCTT  600
AATAGCCCCCCTTGGTCCCTGCTCCTTCTGCTGTCCAAAGGAACTGCATGTAAACTGCAT  660
AGGCTCTTGTCCCCATGTCCCTTTCTTTTGCTGTCATGGTGTTTTGGAGTGACGGAACGG  720
TCGTACAACCAGATGCTCGTGGAGGCCCCTGGGAGCCGGAGAACTTCGAATCTTCCTGTC  780
TT                                                            782
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Fig. 1. Complete nucleotide (782 bp) and deduced amino acid (149 amino acids) sequences of the Calmodulin from the olive flounder *Paralichthys olivaceus*. The predicted ORF is from 82 to 531 bp (start and stop codons are shown in bold). The four Ca2+-binding motifs (EF-I, -II, -III, and -IV) are shown in the box.

A computer search using BLSTP revealed that the deduced primary sequence of CaM cDNA contains four Ca2+-binding motifs known as the helix-loop-helix EF-hands (EF-I, EF-II, EF-III, and EF-IV). Alignment analysis revealed that the amino acid sequence in the second, third, and fourth EF-hands are more conserved than that in the first EF-hand domain compared with others (Fig. 2). It has been reported that phosphocalmodulin is found in cells and tissues and the activity of calmodulin is altered by mitogen-mediated phosphorylation [17]. Olive flounder CaM also possesses several putative phosphorylation sites predicted by NetPhos 2.0 Server [1], which include three serine (Ser18, Ser82 and Ser102), three threonine (Thr29, Thr80 and Thr118) and one tyrosine (Tyr100) residues. These potential phosphorylated residues may affect the interaction of CaM with target proteins. Moreover, Thr80 and Ser82 are located in the central α-helix of CaM, a region important for its interaction with CaM-dependent protein targets.

Expression analysis of olive flounder CaM

To examine the tissue distribution profile of the *CaM* gene, total RNA was extracted from the brain, muscle, intestine, kidney, skin, fin, gill and eye of normal olive flounder.

Semi-quantitative PCR analysis revealed that the CaM mRNA is expressed highly in brain and intestine, and moderately in kidney, gill, and eye of healthy olive
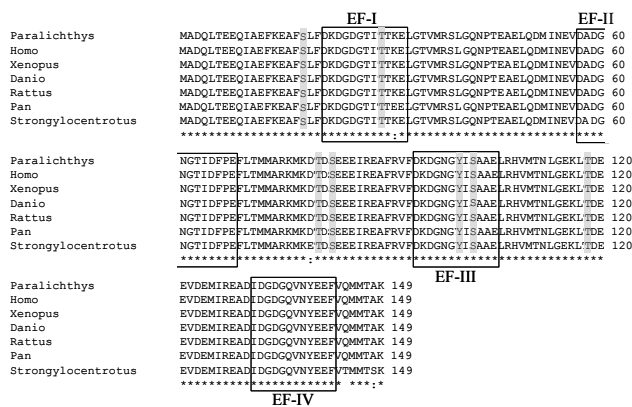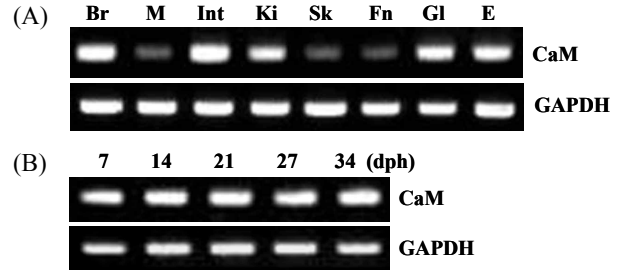

Fig. 3. Expression of the *CaM* mRNA. (A) RT-PCR was performed on equal amounts of total RNA isolated from the internal organs of adult fish. B, brain; M, muscle; I, intestine; K, kidney; Sk, skin; F, fin; G, gill; E, eye. (B) RT-PCR was performed on equal amounts of total whole-body RNA isolated at different developmental stages. The time points are expressed as days post-hatching (dph). *GAPDH* was used as an internal control.

flounder (Fig. 3A). This result is consistent with expression pattern of CaM in other vertebrate. In mammalian, CaM genes are expressed at a particularly profound level in the central nervous system (CNS), especially in the highly polarized neurons. The rodent CaM genes are expressed in a gene-specific manner in the developing and adult brain under physiological conditions [14,15]. Hoenderop *et al.* [7] reported that intestinal Ca^{2+} absorption by Ca2+-binding proteins is a crucial control system in the regulation of Ca^{2+} homeostasis

In addition, to determine at which stage of development *CaM* is transcribed, its expression was analyzed by PCR at 7, 14, 21, 27, and 34 days post-hatching (dph). The *CaM* transcript was detected early in development and slightly increased until 34 dph (Fig. 3B). CaM protein is used to support embryonic development [14]. CaM null mutant animals proceed through embryogenesis normally but die soon after hatching with physiological defects. Taken together, CaM may be necessary for early olive flounder development and that it may have a part in homeostasis.

요 약

Calmodulin은 Ca^{2+} 결합단백질로서 생체 내에서 Ca^{2+} 의존적 기작을 통하여 다양한 생물학적 기능에 관여한다. 본 연구에서는 넙치 *Paralichthys olivaceus* 의 cDNA library로부터 Calmodulin cDNA를 분리 동정하였다. 염기 서열 및 아미노산 서열을 분석한 결과, 넙치 Calmodulin cDNA는 782개의 nucleotides 로 구성되어 있고, 4개의 잘 보존된 Ca^{2+}결합 motifs (EF-I, EF-II, EF-III, EF-IV)를 가지는 149개의 아미노

Fig. 2. Multiple alignment of the amino acid sequences of the *CaM* Ca2+-binding motifs with those of *Rattus norvegicus* (GenBank accession no. NP_114175), *Homo sapiens* (NP_008819), *Danio rerio* (NP_998516), *Xenopus laevis* (NP_001080864), *Strongylocentrotus purpuratus* (XP_7808 62) and *Pan troglodytes* (BAF62378). The four Ca2+-binding motifs (EF-I, -II, -III, and -IV) are shown in the box. Putative phosphorylation sites are shown in gray boxes.

산 잔기를 전사할 수 있는 open reading frame을 포함한다. 또한 번역된 아미노산 서열은 인간, 쥐, zebra-fish, 개구리의 Calmodulin 아미노산 서열과 100% 동일성을 보이며 보라성게, 침팬지의 Calmodulin 아미노산 서열과 각각 97, 99%의 동일성을 보인다. 넙치 Calmodulin 전사체는 뇌와 장 조직에서 많은 양이 발현되었고, 신장, 아가미, 눈, 근육, 피부, 지느러미에서도 발현이 관찰되었다. 또한 넙치 Calmodulin 전사체는 수정 후 7일째의 발생 초기 단계 시료에서도 발현되어 수정 후 34일째까지 그 발현이 서서히 증가하였다. 이상의 결과들로부터 넙치 Calmodulin은 넙치의 발생 초기 단계에서 필요한 단백질로 생각되며 아마 항상성 유지에 중요한 역할을 할 것으로 예상된다.

Acknowledgments

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