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cDNA Cloning and Tissue Distribution of Two Parvalbumin Isoforms from the Hermaphrodite Fish *Rivulus marmoratus* (Cyprinodontiformes, Rivulidae)

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We isolated two parvalbumin cDNAs by expressed sequence tag analysis (1,577 ESTs in total) from the self-fertilizing fish *Rivulus marmoratus* (Cyprinodontiformes, Rivulidae). Two isoforms of parvalbumin genes showed high similarity to those of carp at 88% and 91% amino acid residues identity, respectively, and showed 79.8% similarity between two parvalbumin isoforms. Of 1,577 ESTs from *R. marmroatus* sequenced, parvalbumin 1 gene was most abundant. This gene was strongly expressed in the order of muscle, eye, and brain, while it was expressed slightly in other tissues. In this paper, we discussed on the *R. marmoratus* parvalbumin genes on its sequence and basic characteristics.

Keywords: *Rivulus marmoratus*, Parvalbumin, EF-hand calcium binding protein, Fish

Introduction

Parvalbumins, Ca^{2+}-binding proteins with a low molecular weight, are usually detected in relatively high amounts in white muscle of lower vertebrates (Van Do et al., 2003), and were recognized as a Ca^{2+} buffer to play a role not only as a soluble muscle relaxing factor (Huriaux et al., 2002; Nelson et al., 2003) but also as a cytosolic Ca^{2+} ion reservoir (Pauls et al., 1996). It involves in the protection of non-muscle cells, particularly neurons, against calcium-induced damage (Heizmann and Braun, 1992; Van do et al., 2003).

Teleosts are known to have 2 to 5 isotypes of parvalbumins (Huriaux et al., 2002). Each isotype of parvalbumins plays a specific role in muscle activity on developmental stage (Huriaux et al., 2002). Fish parvalbumins also has been considered as major allergens mediated by IgE antibodies (Hamada et al., 2003; Van Do et al., 2003) which becomes a major concern in cross-allergenicity and its clinical significance (Swoboda et al., 2002a; Swoboda et al., 2002b). The allergenicity has been reported in various fish species including cod *Gadus callarias* (Elsayed and Aas, 1970), Atlantic salmon, carp, goldfish (Nelson et al., 2003), mackerel, medaka, zebrafish (Hsiao et al., 2002), and catfish. However, the transcriptional regulation of fish parvalbumin gene has been poorly studied, even though several parvalbumin genes has been isolated from at least 7 fish species as reported above.

The hermaphroditic fish *Rivulus marmoratus* (Cyprinodontiformes, Rivulidae) is a model species for various researches such as molecular and environmental biology (Lee et al., 2002; Kim et al., 2004a; Kim et al., 2004b; Lee et al., 2005a; Lee et al., 2005b), genetics (Lee et al., 2001; Lee and Lee, 2002; Li et al., 2004), and other aspects. Thus, we initiated to develop this species as a model for multipurpose genomic study. In this paper, we report sequence information and tissue-specific expression of two different parvalbumin genes from the hermaphroditic fish *R. marmoratus*. The present advance provides the fundamental baseline data for better understanding on the function of fish parvalbumin especially with repspect to the genomic perspectives.

Materials and Methods

Fish, RNA extraction and construction of unidirectional cDNA library from *R. marmoratus*

The hermaphroditic fish *Rivulus marmoratus* (adult ≈3-5 cm) were raised in aquarium at the Department of Environmental Science, Graduate School, Hanyang University, Seoul in South Korea. Whole body tissues were used for total RNA extraction from adult fish (3 years old). To extract total RNAs, the fishes were frozen in liquid nitrogen prior to RNA

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extraction. The whole body tissue was firstly ground with a pestle and then homogenized in 3 volume of TRIZOL with a hand-hold tissue grinder. A total RNA was extracted by manufacturer's suggestion. To purify mRNA from total RNAs, we used mRNA purification kit (Invitrogen, USA), followed manufacturer's suggestion, and kept isolated mRNA at -70°C until use.

To analyze ESTs from *R. marmoratus*, we constructed cDNA library with mRNA extracted from whole body tissues. To make unidirectional cDNA library, *R. marmoratus* λZAP II cDNA library packaing kit (Stratagene, USA) was constructed. To synthesize cDNAs, we followed manufacturer's suggestion. To make unidirectional cloning, we used linker and adaptor that have *Eco*RI and *Xho*I restriction enzyme sites. Packaging and the titration of primary λ recombinant phages was followed by manufacturers' suggestion.

Conversion of *R. marmoratus* λ ZAP II cDNA library to phagemid, cDNA cloning of *R. marmoratus* parvalbumin genes and comparison to other homologues and the protein motif search

The small quantity (3-4 μl) of *R. marmoratus* λ ZAPII cDNA library was taken and subjected to *in vitro* conversion to phagemid DNA with help of helper phage and *E. coli* SOLR® strain. Colonies were randomly picked up for culture to identify its inserts. After cutting the inserts with *Eco*RI, the recombinant phagemid DNAs over 1.0 kb were subjected to sequence analysis. Sequencing analysis was done with automated sequencer ABI 377 (PE Biosystems Co., USA). The *R. marmoratus* ESTs were compared to the GenBank database to identify the closest homologues, and registered to the *R. marmoratus* EST database (data not available on public yet).

The ESTs of *R. marmoratus* were collected by random sequencing analysis. One of most expressed genes was picked and searched *R. marmoratus* EST database to isolate another related homologues. The DNA sequences isolated were analyzed using the computer program DNASIS version 3.2 (Hitachi Software Engineering Co. Ltd). To search protein motif, we used the website of EBI (http://www.ebi.ac.uk/ppsearch/).

Tissue-specific expression of *R. marmoratus* parvalbumin genes

To analyze the expression of *R. marmoratus* parvalbumin gene, we sampled several tissues such as brain, eye, gonad, intestine, liver, skin, and muscle. Total RNAs were extracted from tissues stored at -80°C prior to use. We used the two RT-PCR primers, Rm-parvalbumin 1 (Rm-Parv1-F, 5'-GCA AAC ACA CCA GAG AAC AAA-3'; Rm-Parv1-R, 5'-TTA TGC CTT AAC CAT GAC AGC-3'; expected product 350 bp) and Rm-parvalbumin 2 (Rm-Parv2-F, 5'-AGA TAA CTT CAA GTT AAC AAA-3'; Rm-Parv2-R, 5'-TTA ACC CTT GAC CAA GGC AGC-3'; expected product 350 bp). Internal control primers (Rm-GAPDH-F 5'-CGG TAA GCT GTG GAG GGA CGG CCG CG-3', Rm-GAPDH-R 5'-TGG TGC TCG GTG TAT CCC AGA ATG CC-3'; expected product 265 bp; GenBank accession number AY864771) were used with Rm-parvalbumin primers in the same reaction to control for the amount of template first-strand cDNAs. We carried out RT-PCR (1 μl of first-strand cDNA, 5 μl of 10 × LA buffer, 8 μl of 2.5 mM dNTPs, 5 μl of 25 mM $MgCl_2$, 100 pM each primer, 0.5 μl of LA *Taq* polymerase (TaKaRa); total volume 50 μl) with three different cycles (1 cycle, 95°C, 5 min; 35 cycles, 98°C, 25 sec, 50°C, 1 min, 72°C, 1 min; 1 cycle 72°C, 10 min) by iCycler (Bio-Rad). The relative level of gene expression was quantified after we checked the intensity of the ethidium bromide-stained bands from *R. marmoratus* parvalbumin and GAPDH genes. These experiments were carried out in triplicate and the Student's t-test was applied for statistical analysis.

Results and Discussion

Of 1,577 ESTs initially sequenced, most abundantly expressed gene was *R. marmoratus* parvalbumin 1 gene (6.53 %). It was more strongly expressed rather than those of key structural genes of cells such as β-actin gene (3.1%). Thus, *R. marmoratus* parvalbumin gene was most abundant transcripts in *R. marmoratus* ESTs. This kind of transcript such as strong expression of Ca^{+2}-associated proteins was shown in ESTs of the intertidal copepod *Tigriopus japonicus* (Lee et al., 2005). Perhaps Ca^{+2}-binding associated proteins would be important to maintain the homeostasis in tissues.

Of 1,577 *Rivulus* ESTs, ten different transcripts of parvalbumin genes were collected, which were categorized into two different parvalbumin genes (parvalbumin1 and 2) as shown in Fig. 1 and Fig. 2. These patterns were similar to those of other fishes, indicating it would have similar effects as a major allergen to human. Two *R. marmoratus* parvalbumin genes were encoded 109 amino acid residues, and showed 79.8% similarity each other. We registered them to the GenBank under the name of AY682949 (*R. marmoratus* parval-

***R. marmoratus* parvalbumin 1**

```
   1 GCACGAGGCTTGTCTGTGTCATCTGTACCCTACAAAGCAAACACACCAGAGAACAA
  57 AATGGCTTTCGCAGGTGTACTGAATGATGCTGACATCACCAAAGCCCTGGATGAGT
   1  M  A  F  A  G  V  L  N  D  A  D  I  T  K  A  L  D  E  C
 113 GCAAAGGCGCTGACTCATTCGACTACAAGAAGTTCTTCAAGACATGCGGTCTGGCC
  20  K  G  A  D  S  F  D  Y  K  K  F  F  K  T  C  G  L  A
 169 GGAAAGAGCGCCGATGATGTCAAGAAGGCCTTCGCCATCATTGACCAGGACAAGAG
  38 G  K  S  A  D  D  V  K  K  A  F  A  I  I  D  Q  D  K  S
 225 TGGCTTCATTGAGGAGGATGAGCTGAAGCTGTTCCTGCAGAACTTCAGTGGAGGTG
  57  G  F  I  E  E  D  E  L  K  L  F  L  Q  N  F  S  G  G  A
 280 CTCGCGCCCTGACCGACGCTGAGACCAAGGCTTTCCTGAAGGCCGGCGACAGCGAC
  76   R  A  L  T  D  A  E  T  K  A  F  L  K  A  G  D  S  D
 337 GGTGATGGCAAAATTGGAGCTGATGAGTTCGCTGTCATGGTTAAGGCATAAGACAG
  94 G  D  G  K  I  G  A  D  E  F  A  V  M  V  K  A  *
 393 CAACTGACCAACAAACACCTGCTCTAATGGAACAACAAAGCCCACGTCGACCTCCC
 448 CCTTTTCATCTGAATATAAATAATTTTTATACATTTGCTTAAGGGAGTTTCCTCCT
 505 CTATGCAACACACTGTCCAGAAGTGATGATTGTAAATGTCGCTCGGTTGTGTTCAT
 561 GTCTTAAATATGAATTTTGCTAACTGTAAAATCTATAATGCACTTTCCAGGAACTA
 617 AAGGGTGAAAGAAAATAAACATTCTTTTGCTGCGGTCTTAAAAAAAAAAAAAAAAA
 673 AAAAAAAAAAAAAAAA
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***R. marmoratus* parvalbumin 2**

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   1 ACGAGGAGCGAATCCTCTCTAGCCTTAGAGATAACTTCAAGTTAACAAAATGGCAT
   1                                                  M  A  F
  57 TCGCCGGACTTCTGAGTGATGCTGACATCACTGCAGCCCTCGCAGCCTGCCAAGCT
   4   A  G  L  L  S  D  A  D  I  T  A  A  L  A  A  C  Q  A
 113 GCTGACTCTTTCAAGCACAAGGAGTTCTTCGCCAAGGTCGGCCTGGCCGGCAAGTC
  22 A  D  S  F  K  H  K  E  F  F  A  K  V  G  L  A  G  K  S
 169 TGCCGATGACATCAAGAAGGCCTTTGCTGTCATTGACCAGGACAAGAGCGGCTTCA
  41  A  D  D  I  K  K  A  F  A  V  I  D  Q  D  K  S  G  F  I
 225 TTGAGGAGGATGAGCTGAAACTGTTCCTGCAGAACTTCTCCGCCAGCGCCAGAGCT
  60   E  E  D  E  L  K  L  F  L  Q  N  F  S  A  S  A  R  A
 281 CTGACCGACGCTGAGACAAAGACCTTCCTGAAGGCCGGTGACTCTGATGGTGACGG
  78 L  T  D  A  E  T  K  T  F  L  K  A  G  D  S  D  G  D  G
 337 CAAGATCGGAGTTGATGAGTTCGCTGCCTTGGTCAAGGGTTAAACGAACAAAGTGA
  97  K  I  G  V  D  E  F  A  A  L  V  K  G  *
 393 CCAACATCTGACTTCACTTCTCTAAAGGAACAACTTGAGCCAAGACATACTCTCTT
 449 CTTCTCCTCTTCCCACTCACTCTTTCTCGTAGACTCTCTGAAGCACTTTTGTACAC
 505 TTGACCTTTGACCGCCCACAGACAACCCACTCTTTCTCTCTCTCAGGCAGTGCTCA
 561 CCCCAGCGTGATCCATGACGAGTGTACCTGACTAACTGTATGGCGTGACTGACTTG
 617 TGTCTTTCTCCCTCTCTCTCTCTCCCTCCCTCTCCCTCTCTTTCTCTCTCTCTC
```

Fig. 1. Representative cDNA sequences of *Rivulus marmoratus* parvalbumin 1 and 2 genes. The poly (A) termination sequence (AATAAA) was underlined.

```
Rivulus1                MAFAGVLNDADITKALDECKGADSFDYKKFFKTCGLAGKSADDVKKAFAI
Mackerel                ....S..K..EV.A...G..A.G...H.....A...S...T.E.......
Rivulus2                .....L.S.....A..AA.QA....KH.E..AKV.........I.....V
Carp-1.01               .....I.......A..QG.QA.......S..AKV..SA.TP..I.....V
Carp-1.02               ............A..EA..A....NH.T..AKV..TS..............
AtlanticsalmonB-X97825  .S...LN-...VAA..AA.TA....NH.A..AKV...S..S.......YV
AtlanticsalmonB-X97824  ..C.HLCKE...KT..EA..A..T.SF.T..H.I.F.S..........KV
                        *: * :  :*::  **  * .*.:*..* **   *::.*:.*::**** :

Rivulus1                IDQDKSGFIEEDELKLFLQNFSGGARALTDAETKAFLKAGDSDGDGKIGA
Mackerel                ..........E.........KA.....S.....................I
Rivulus2                ..........................AS.....T.....................V
Carp-1.01               ..........................A.....................V
Carp-1.02               ..........................KA.....G...T.....................V
AtlanticsalmonB-X97825  ..........................AS.....AD..K....M..V
AtlanticsalmonB-X97824  ...A.....VE.........CPK..E.....A....M..I
                        **** ***** :********* ** *:*.***:** ** **** **

Rivulus1                DEFAVMVKA (109 aa)
Mackerel                ...A.I.G (109 aa)
Rivulus2                ...AL..G (109 aa)
Carp-1.01               ...AL... (109 aa)
Carp-1.02               ..TAL... (109 aa)
AtlanticsalmonB-X97825  ...A.I.G (109 aa)
AtlanticsalmonB-X97824  ...L..Q (109 aa)
                        ***:.::*
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Fig. 2. Comparison of amino acid residues of fish parvalbumin. The symbols "*", ":" and "." indicate the identical and the strong or moderate positive residues of substitution between fishes, respectively. Boxes indicate the Ca^{+2}-binding motif.

bumin 1) and AY682950 (*R. marmoratus* parvalbumin 2), respectively.

After we checked the ClustalW analysis and protein motifs (http://www.ebi.ac.uk/ppsearch) of fish parvalbumin genes from Atlantic salmon (Lindstrom et al., 1996; Van Do et al., 1999), carp (Bugajska-Schretter et al., 1999; Swoboda et al., 2002b) and mackerel (Van Do et al., 1999), we found two EF-Ca^{+2} binding protein motif as marked with boxes as shown in Fig. 3. This suggests that *R. marmoratus* parvalbumin proteins would play as Ca^{+2}-associated role in cells as shown in other fishes that would be enable to show a high affinity for Ca^{2+} and Mg^{2+} ions at physiological pH and ionic strength (Hsiao et al., 2002).

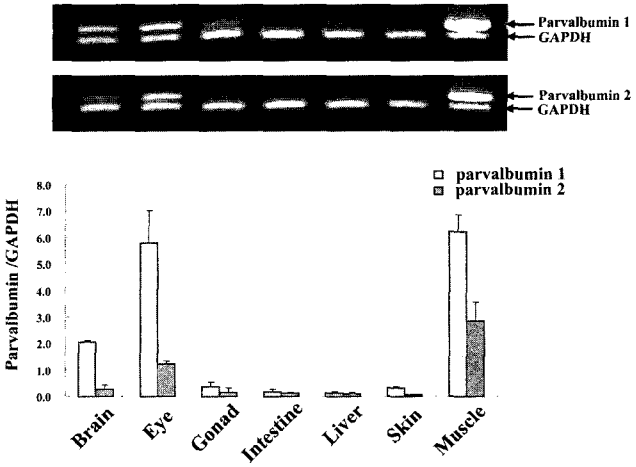

Fig. 3. Tissue-specific expression of *R. marmoratus* parvalbumin 1 and 2 genes.

As already shown in Fig. 1, we cloned two different *R. marmoratus* parvalbumin genes and they showed two different clades in phylogeny (data not shown). In general, parvalbumins are subdivided into two phylogenetic lineages (α-type and β-type), and are able to be distinguished by their different isoelectric points (α>pI 5, β<pI 5) and cell-type specific expression (Van Do et al., 1999). On molecular phylogenetics of fish parvalbumin genes, to our knowledge, they have two duplicated genes as appeared in carp, while in other fishes such as mackerel and Atlantic salmon there is one kind of parvalbumin. In the level of gene expression, *R. marmoratus* parvalbumin 1 gene is more abundantly expressed based on EST data rather than other transcripts, and had pI 4.59 with molecular weight 11.6 kDa, while *R. marmoratus* parvalbumin 2 had pI 4.64 with molecular weight 11.4 kDa. In case of *R. marmoratus* parvalbumins, they showed a lower pI value as expected. This would be derived from species specificity of parvalbumin proteins.

On expression of *R. marmoratus* parvalbumin 1 and 2 genes, they were strongly expressed in muscle, eye and brain in order, respectively. Two *R. marmoratus* parvalbumin genes showed the same patterns of expression, although parvalbumin 1 was expressed more strongly rather than that of parvalbumin 2. These data supported that parvalbumins were abundantly expressed in muscle and nerve-related tissues for getting some role of Ca^{+2} reservoir and Ca^{+2}-induced damage, respectively (Heizmann and Braun, 1992; Pauls et al.,

1996; Huriaux et al., 2002; Nelson et al., 2003; Van Do et al., 2003). Recently, Yang et al. (2004) reported that expression of parvalbumin α and β gene is differentially regulated in the rat organ of corti during development. We also checked expression of *R. marmoratus* parvalbumin 1 and 2 gene in larvae (head and trunk) and adult (head and trunk) but they were not shown different expression patterns (data not shown).

We also checked expression patterns of *R. marmoratus* parvalbumin in terms of temperature shift, as Nelson et al. (2003) reported that cold-acclimation was raised expression of Ca^{+2}-associated genes such as parvalbumin β in goldfish but we were not able to find any difference of expression of *R. marmoratus* parvalbumin 1 and 2 gene between 15°C- and 25°C-acclimated *R. marmoratus* (data not shown). This also may indicate the fact that species difference would be existed in expression of parvalbumin genes.

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