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Molecular Studies on the Disease Resistance Gene, Proopiomelanocortin (POMC), from Flounder (*Paralichthys olivaceus*)

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Proopiomelanocortin (POMC) plays an essential role in the disease resistance system and is the precursor protein of biologically active peptides such as adrenocorticotropin (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-melanocyte-stimulation hormone (β-MSH) and β-endorphin. We have isolated and sequenced two different forms of POMC cDNA, POMC-I and POMC-II, from a pituitary cDNA library of flounder. POMC-I cDNA consisted of 956 bp corresponding to deduced amino acids of 216 residues and POMC-II cDNA was 982 bp in length corresponding to 194 amino acids, respectively. The results of deduced amino acids analysis of the clones showed high sequence homology with previously reported POMCs amino acid sequences from various species. The homology between flounder POMC-I and -II is 57% identity. We also constructed a phylogenetic tree based on POMC amino acid sequences.

Key words: Flounder, *Paralichthys olivaceus*, Molecular cloning, Proopiomelanocortin

Introduction

Proopiomelanocortin (POMC) is well known as a stress response hormone and involved in the disease resistance system and much of its biological and physiological characteristics have been studied in mammals so far (Groenink et al., 1994). POMC is a multifunctional precursor protein which generates several biologically active peptides including adrenocorticotropin (ACTH), α-melanocyte stimulating hormone (α-MSH), β-melanocyte stimulating hormone (β-MSH), and β-endorphin by limited proteolysis (Roberts and Herbert, 1977). Mostly a cleavage site is single or paired basic amino acids (Theodore et al., 1994).

The nucleotide and amino acid sequences of POMC gene were determined from various species including mouse (Uhler et al., 1983), guinea pig (Keightley et al., 1991), chum salmon (Heierhorst et al., 1990), sockeye salmon (Okuta et al., 1996), rainbow trout (Tollemer et al., 1997), carp (Arends et al., 1998), and human (Whitfeld et al., 1982) and revealed that the structure of POMC from various species has been highly conserved, especially in ACTH, α-MSH, and β-endorphin. In addition to that, some amphibians and fish species were thought to be undergone chromosome duplication having two POMC genes. Some teleosts including common carp, chum salmon, rainbow trout, and a chondrost like white sturgeon have shown two POMCs in fish species (Alrubaian et al., 1999). Among them, the rainbow trout has a unique C-terminal extension of 25 amino acid in POMC-A and there was a great evidence reported as a new neuropetide derived from POMC-A (Tollemer et al., 1999). Furthermore, there was an evidence for functional differences between the two POMCs in rainbow trout in that only POMC-A was expressed in sexually inactive fish (Salbert et al., 1992). There were several studies reported that the expression of two different POMCs was also detected in organs or

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tissues including the genital tract (Pintar et al., 1984), the placenta (Liotta et al., 1980), and immune system in mammals (Harbour et al., 1987).

In the present study, we isolated and characterized two different cDNAs encoding flounder (*Paralichthys olivaceus*) POMCs that might have important implication in the understanding the fish central nervous system and their crucial metabolism involved in disease resistance system, and also to initiate studies on the regulation of POMC gene expression in response to stressful stimuli which relates in various disease. We describe here sequence analysis of the cloned flounder POMC cDNAs with other species and also a phylogenetic tree constructed based on POMC amino acid sequences.

Materials and Methods

Materials

The *E. coli* strain XL1-Blue was used for library titration, transformation, and color selection. The primers used for this study are summarized in Table 1. Restriction enzymes, reverse transcriptase, and Ribonuclease inhibitor were purchased from Promega (U.S.A.). The twenty adult flounders alive were purchased from a local seafood market and held in a seawater tank with circulation and aeration at 15℃ until used.

Table 1. Oligonucleotide primers used for the isolation of flounder POMC cDNAs

Primer	Direction	Sequence	Remark
POMCf-1	forward	5'-A(T/C)TCCATGGA(G/C)CACTTCCG-3'	degeneracy primer
POMC-IIR	reverse	5'-TAGGAGCGCTTGGCCTGAGG-3'	specific primer for POMC-II

RNA isolation and construction of cDNA library

The preparation of total RNA from flounder pituitary tissue was performed with total RNA isolation kit (Promega). Complementary DNA library was constructed as described in the Manufacturer's instruction (Stratagene). Messenger RNA was isolated from flounder pituitary tissue using oligo (dT) cellulose. Flounder cDNA was synthesized with MMLV-reverse transcriptase and second-strand cDNA was synthesized with DNA polymerase and RNase H. Synthesized cDNA was packaged with the GigapackIII gold packaging extract kit (Stratagene, U.S.A.).

Screening of POMCs from flounder cDNA library

Conserved sequences of POMCs among fish species were determined and used for the preparation of degeneracy primer (Table 1). PCR was carried out using these primers as described in (Kim et al., 1997; Cho et al., 1999). Probe was labeled with DIG (Digoxigenin)-oligonucleotide 3'-end labeling kit (Boehringer Mannheim). Positive plaques were screened with above probe and further confirmed by the second screening as descrtibed in (Sung et. al., 1999; 2000).

In vivo excision and DNA Sequencing

Positive plaques were recovered and the phagemid containing the insert was excised according to the manufacturer's instructions (Stratagene, U.S.A.). Sequencing of the excised phagemid was performed by the method described in (Kim and Richardson, 1993; 1994) and ABI 310 Genetic Analyzer according to the manufacturers instructions (Perkin Elmer, U.S.A.).

Results and Discussion

In the present study, we have identified two types of POMC genes, POMC-I and POMC-II, from flounder. Some fishes and amphibians often have two genes for a certain hormone, probably due to their evolutionary process based on tetraploidization millions years ago. So far, two POMCs had been cloned from sockeye salmon (Okuta et al., 1996), chum salmon (Heierhorst et al., 1990), common carp (Arends et al., 1998), and rainbow trout (Tollemer et al., 1997).

We have cloned two POMC genes using a flounder pituitary library. Two PCR products, about 800 bp and 700 bp, were obtained by PCR using POMCf-1 and T7 promotor primers. Both PCR products represented POMC genes but there were much of sequence differences between two clones, suggesting two different POMC genes. Consequently, using the probes made with both PCR products, we obtained several positive clones and analyzed their nucleotide sequences. The results

showed that they were two types of POMC genes, POMC-I and POMC-II from flounder. POMC-I with a full ORF gene was isolated but only a partial POMC-II was cloned. In order to isolate the full length clone, which contains the longest insert gene corresponding to POMC-II, colony selective PCR was performed with POMCr-II and T3 promotor primers. The longest clone with full ORF and poly A tail sequences was composed of the nucleotide sequence of 956 bp for POMC-I (Fig. 1) and 982 bp for POMC-II (Fig. 2). Deduced amino acid sequences were 216 (POMC-I) and 194 (POMC-II) residues and the homology between POMC-I and -II is 57% identity. Interestingly, two different POMC clones still share common active peptides such as ACTH, α-MSH, and β-endorphin. Neither did contain γ-MSH like other teleost POMC, whereas ACTH, α-MSH, β-MSH, and β-endorphin were present.

The sequence of the cloned cDNA was aligned with NCBI nucleotide sequence data base using a Blast program and showed high sequence identity with previously reported POMCs. As shown in Figure 3, the structural comparisons of the deduced amino acids sequences of flounder POMCs were

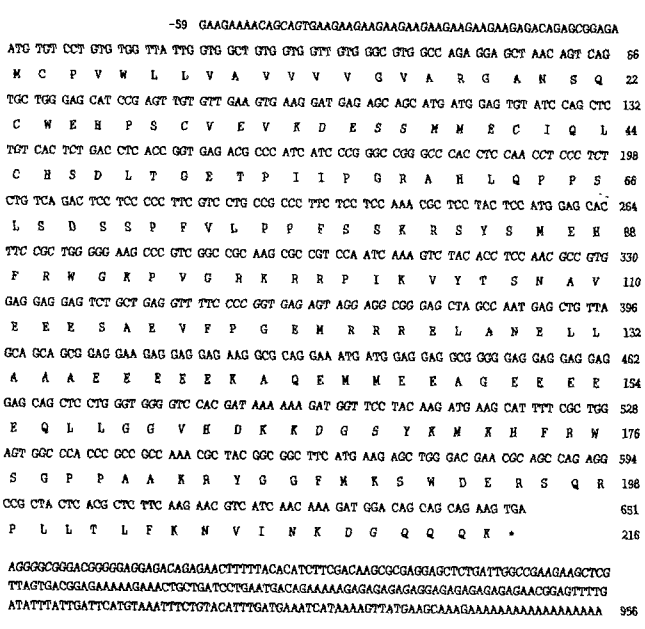

Fig. 1. Nuleotide and deduced amino acid sequences of POMC-I cDNA from flounder pituitary cDNA library. The number of the encoded nuleotide and deduced amino acid sequences shows at the end of each lane.

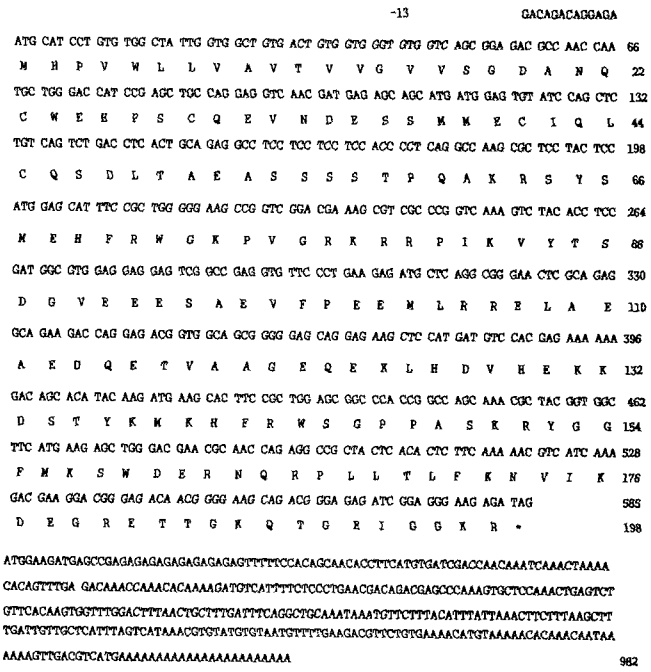

Fig. 2. Nuleotide and deduced amino acid sequences of POMC-II cDNA of flounder.

conducted with the amino acids sequences of carp, trout, rat, and human using Clustal Multiple Alignment Program (Higgins and Sharp, 1989). Comparison of POMC amino acid sequences to various species revealed that biologically active peptides such as α-MSH, β-MSH, ATCH, and β-endorphin were highly conserved whereas other sequences were less conserved. In addition, they still had some variant in amino acid sequences within well conserved sequences (Fig. 3). Interestingly, there were two interesting differences in flounder POMCs from other fish POMCs. One is that POMC-II has extended 11 amino acid residues suggesting another type of hormonal peptide like POMC-A of rainbow trout, and the second is that there are little joining sequences between biologically active peptides.

Fig. 4 shows the phylogenetic tree based on the homology of the amino acid sequences of POMCs compared in Fig. 3. The amino acid sequence identity was calculated using Genedoc program. Their gaps were also considered and the highest and the lowest scores represented in the ways of raw score and percent scores. Tree view and Genedoc program were used to make a phylogenetic tree. The identity of two flounder POMCs was as low as 57% like rainbow trout POMCs (46%) whereas carp and

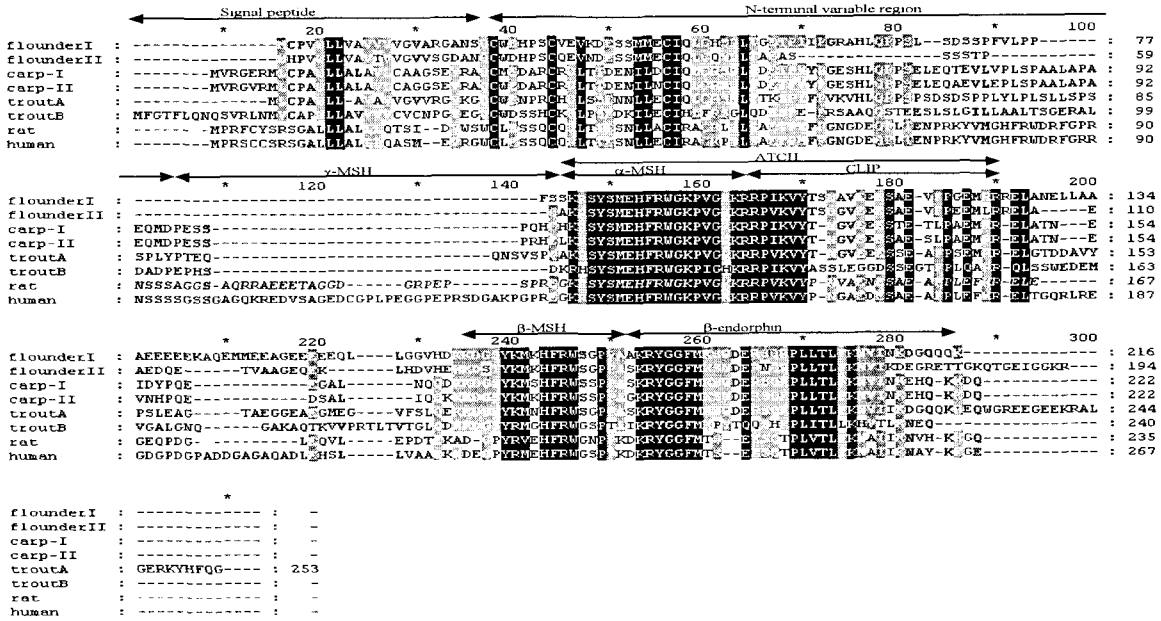

Fig. 3. **Comparison of the deduced amino acid sequences of POMCs from flounder, carp, trout, rat, and human.** The sequence of the flounder POMCs is aligned by using Clustal Multiple Alignment Program (Higgins and Sharp, 1989) with carp, trout, rat, and human. Asterisks indicate a perfect match between all the sequences. Dots indicate a conversative replacement.

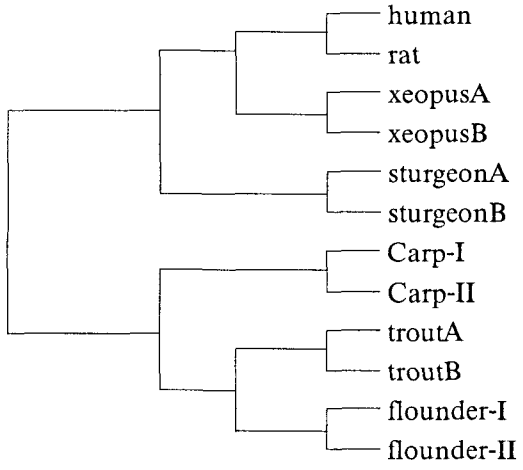

Fig. 4. **Phylogenetic tree of the POMCs based on the amino acid sequences.** All the amino acid sequences were from NCBI database. Tree view and Genedoc program were used to make a phylogenetic tree. The long distance indicate low relationship among species.

sturgeon showed 89% and 90% identity, respectively. In addition, there were few gaps between two POMCs in carp and sturgeon but flounder and rainbow trout had frequent gaps within amino acid sequences. The long distance indicate low relationship among species. This advance in our knowledge holds significant implications in the understanding of the critical roles of the POMCs involved in disease resistance system, and also in initiating studies on the regulation of POMC gene expression in response to stressful stimuli which relate in various disease.

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