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Functional relevance of three proopiomelanocortin (POMC) genes in darkening camouflage, blind-side hypermelanosis, and appetite of *Paralichthys olivaceus*

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ABSTRACT

To determine whether proopiomelanocortin (POMC) genes are involved in darkening color camouflage, blind-side hypermelanosis, and appetite in flatfish, we isolated and cloned three POMC genes from the pituitary of the olive flounder (*Paralichthys olivaceus*) and compared their amino acid (aa) structures to those of POMC genes from other animals. Next, we examined the relationship of these pituitary POMC genes to camouflage color change, blind-side hypermelanosis, and appetite by quantifying mRNA expression. Olive flounder (*of*)-POMC1, 2, and 3 cDNAs consisted of 648-bp, 582-bp, and 693-bp open reading frames (ORF) encoding 216 aa, 194 aa, and 231 aa residues, respectively. Structurally, the three *of*-POMC cDNAs consisted of seven peptides (signal peptide, N-POMC, α-MSH, CLIP, N-β-LPH, β-MSH and β-END [or END-like peptide]) that are similar to those of other fish POMC cDNAs. α-MSH encoded a protein composed of 13 aa and β-MSH encoded a protein composed of 17 aa. The three POMC genes were predominantly expressed in the pituitary gland, but they were also expressed in a variety of tissues, including brain, eye, kidney, heart, testis, and skin. *of*-POMC2 exhibited the highest expression, while *of*-POMC3 displayed the lowest expression. The relative levels of *of*-POMC1 and 3 mRNAs were not influenced by background color and feeding (or fasting), but the relative level of *of*-POMC2 mRNA significantly increased in response to a dark background and fasting. The relative levels of *of*-POMC1 and 2 mRNAs were significantly higher in hypermelanic fish; however, we did not determine a direct anorexigenic or orexigenic relationship for the three POMC genes. These results indicate that pituitary POMC genes are related to darkening color change and the differentiation of pigment cells, but they are not directly related to appetite.

1. Introduction

Proopiomelanocortin (POMC) is a common precursor of adrenocorticotropic hormone (ACTH), melanophore-stimulating hormone (α-, and β-MSH), and endorphin (END). As in other vertebrates, teleost POMC genes are expressed mainly in the pituitary gland. POMC genes undergo posttranslational processing in which different peptides are generated by two pituitary cell types in the rostral pars distalis (RPD) and pars intermedia (PI): corticotropes or ACTH-producing cells are localized in the RPD, whereas melanotropes or MSH-producing cells are localized within the PI, which is heavily innervated from the neurohypophysis to form the neuro-intermediate lobe (Cerdá-Reverter and Canosa, 2009).

The POMC nucleotide and amino acid (aa) sequences of various animals, including mammals (Lightman and Young Iii, 1988), amphibians (Steveson and Dores, 1996), reptiles (Endo and Park, 2004) and fish (Salbert et al., 1992) have been determined. The inconsistent number of MSH segments in fish POMC suggests that POMC diverged through insertions and deletions of MSH during the evolution of jawed fish. Comparison of the structure of POMC genes clarified that POMC evolved by gene duplication, which increased the number of copies of POMC, and by internal gene duplication and deletion of the MSH domain, resulting in the diversity of POMC with regard to the number of MSHs. The structure of POMC may have diverged through unequal crossing over, which altered the number of MSH segments in POMC, and duplication, which increased the number of copies of POMC during the evolution of vertebrates (Dores and Lecaude, 2005; Sundström et al., 2010; Cerdá-Reverter et al., 2011; Vallarino et al., 2012).

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Also, previous studies demonstrated that fish possess subtypes of POMC genes based on their sequence structure (Okuta et al., 1996; Arends et al., 1998; Alrubaian et al., 1999; Danielson et al., 1999; Takahashi et al., 2005), suggesting that POMC has also diverged through chromosomal duplication, producing a copy of the entire POMC gene through this process. Therefore, flatfishes, including the olive flounder, may have different types of POMC genes in terms of their structure and function. Although many studies on the structure and function of POMC genes have been conducted, the full range of structures and functions remains to be determined. Therefore, it is necessary to clarify whether flatfish species have subtypes of POMC genes and whether functional differences exist among the subtypes due to divergence.

Many studies on the structure of POMC genes have revealed that the MSH segment (α-MSH and β-MSH) is highly conserved among species. In fishes, α-MSH derived from POMC and its receptor (melanocortin receptor: MCR) control physiological color change via melanin granule dispersion in melanocytes in the skin (Thody et al., 1984; Takahashi et al., 2005; Yamanome et al., 2007; Cerdá-Reverter et al., 2011; Mizusawa et al., 2013). α-MSH was previously reported to be related to blind-side malpigmentation, a morphological color change in flatfish (Yamanome et al., 2007); however, no direct evidence has been published concerning the relationship between the POMC peptide and skin color change in flatfish. Moreover, the POMC subtype related to skin color change is unknown. Therefore, it is important to confirm that POMC, a precursor of melanocortin peptide, is related to skin color change and malpigmentation in flatfish, such as the olive flounder (*Paralichthys olivaceus*), a commercially important fish in South Korea.

In mammals, a majority of POMC neurons are located in the arcuate nucleus (ARC) of the hypothalamus (Cone, 2005; Padilla et al., 2012). Moreover, melanocortin receptors are highly expressed in the downstream targets of ARC POMC neurons (Cone, 2005), suggesting that these neurons are a fundamental component in the neural circuit underlying feeding inhibition. The melanocortin system may play an important role in the central regulation of food intake (Kalra et al., 1999; Lin et al., 2000; Pritchard et al., 2002; Murashita et al., 2008; Shimakura et al., 2008; Kojima et al., 2010; Mountjoy, 2010; Saneyasu et al., 2011; Dutia et al., 2012; Zhan et al., 2013) and energy homeostasis (Tritos and Maratos-Flier, 1999; Pritchard et al., 2002; Mountjoy, 2010; Wardlaw, 2011; Pérez Sirkin et al., 2012). In fish, although POMC genes are predominantly expressed in the pituitary gland, they are also expressed in a variety of tissues (gill, heart, kidney, stomach, liver, spleen, muscle, and skin), including the brain (hypothalamus) (Varsamos et al., 2003; Karsi et al., 2004). Examining goldfish, Cerdá-Reverter et al. (2003) demonstrated that positive POMC neurons are exclusively expressed within the mediobasal hypothalamus, indicating that a functional melanocortin system in fishes may participate in the central regulation of food intake. In fish, α-MSH inhibits food intake and is one of several potent anorexigenic neuropeptides in the brain (Lin et al., 2000; Cerdá-Reverter et al., 2003; Shimakura et al., 2008; Kojima et al., 2010; Saneyasu et al., 2011), while β-END, another peptide derived from POMC, stimulates food intake via opioid receptors (De Pedro et al., 1995, 1996). It is not certain that the pituitary POMC subtype genes are not involved in anorexigenic or orexigenic regulation, and there are insufficient data to determine the role of pituitary POMC derivatives in food intake in fish. Thus, it is necessary to clarify the role of pituitary POMC subtype genes with respect to food regulation.

In the present study, we investigated whether the POMC subtypes are present in the flounder, *P. olivaceus*. Next, we surveyed the relevance of pituitary POMC subtype genes to darkening camouflage of ocular skin, morphological color change (blind skin hypermelanosis), and appetite in flounder. First, we examined the primary structure of three POMCs through cloning and peptide analyses. Subsequently, to evaluate the relationship of the pituitary POMC gene expression with darkening camouflage, hypermelanosis, and appetite, we compared the expression of pituitary POMC mRNAs in fish reared in tanks with bright or dark backgrounds, fish reared in tanks with flat or gravel bottoms, and fasting and fed fish.

2. Materials and methods

2.1. Identification of three POMCs

2.1.1. Extraction of total RNA

To isolate the flounder POMC (*of*-POMC) gene, pituitary tissue was removed from adult fish [total length (TL): 31.0 ± 0.22 cm, body weight (BW): 297.7 ± 7.21 g] cultured in indoor running seawater tanks under a natural photoperiod and water temperature. Pituitary tissue sampling was done from flounder anesthetized by immersion in 0.05% 2-phenoxyethanol. Sampled pituitaries were frozen at −80 °C for total RNA extraction. Total RNA was extracted from the sampled pituitaries using a Maxwell 16 LEV simplyRNA Tissue Kit (Cat No. AS1280; Promega, Madison, WI, USA) and a Maxwell 16 instrument. Genomic DNA in extracted matter was eliminated using a recombinant DNase I solution (Cat No. AS1280; Promega) as described by the manufacturer. In addition, using a primer set designed based on the β-actin gene of *P. olivaceus* (GenBank HQ386788), we examined whether the genomic DNA was removed by DNase and was therefore not reverse transcribed by resolving the final PCR product in an agarose gel. No PCR products were observed, indicating that the DNase treatment was effective in removing genomic DNA contamination. Also, we measured RIN (RNA integrity number) values using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc., Germany) with the Agilent RNA 6000 Nano Kit (no. 5067-1511; Agilent Technologies, Inc., Germany) and total RNA content and purity in the extracted matter using a Nanovue plus spectrophotometer (Cat No. 28-9569-65; GE Healthcare, Milwaukee, WI, USA). We confirmed that pure total RNA was stably extracted from pituitary tissue without DNA contamination or RNA degradation.

2.1.2. Synthesis of cDNA, PCR, and RACE

A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE) was used to clone cDNAs encoding three putative POMC genes from olive flounder pituitary glands. The *of*-POMC PCR (clone I) using step I-sense and antisense primers amplified the middle region, including olive flounder POMC cDNA. Based on this nucleotide sequence, the step II-antisense primer was synthesized for 3′-RACE. PCR (clone II) using the step II-antisense primer and 3′-RACE kit primer was used to amplify *of*-POMC cDNA. Based on clones I and II, the step III-sense primer was synthesized for 5′-RACE. Clone III, encoding the *of*-POMC cDNAs, was amplified using the step III-sense primer and the 5′-RACE kit primer. These three PCR-amplified cDNAs were merged to yield the entire sequence of the three *of*-POMC cDNAs (Table 1). Reverse transcription-polymerase chain reaction (RT-PCR) amplification was performed using AccuPower RT/PCR PreMix (K-3035; Bioneer, Daejeon, Korea) with the MyiQ PCR system (Bio-Rad, Hercules, CA, USA) according to the manufacturers' instructions. Prior to PCR, predicted primers (clone I primers) were designed from highly conserved regions of three POMC genes from other animals (Table 1). A PCR mixture containing 1 μg of total RNA was heated at 94 °C for 5 min to activate the enzyme. PCR was composed of 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C, with a final extension for 5 min at 72 °C, according to the PCR kit manual. Amplified PCR products were separated on agarose gels, extracted using the AccuPrep Gel Purification Kit (Cat. No. K-3035; Bioneer), and sequenced by COSMO Gen Tech (Seoul, Korea). For rapid amplification of the cDNA 5′- and 3′-ends (RACE) reactions, RACE target primers for the three *of*-POMCs were newly designed. 3′-RACE primer [5′-CTGTGAATGCTGCGA CTACGAT$(T)_{18}$-3′] and 5′-RACE primer (5′-GTCTACCAG CATTCGCTTCAT-3′) supplied by the CapFishing Full-Length cDNA Premix Kit (Seegene K2000, Seoul, Korea) were used as the antisense and sense primers, respectively (Table 1). Using 1 μg of pituitary total RNA as a template, RACE-ready