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Expression of three proopiomelanocortin subtype genes and mass spectrometric identification of POMC-derived peptides in pars distalis and pars intermedia of barfin flounder pituitary

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Abstract

Proopiomelanocortin (POMC) is a common precursor of adrenocorticotropic hormone (ACTH), melanophore-stimulating hormone (MSH), and endorphin (END). In pituitary gland, POMC receives posttranslational processing by which different peptides are generated in the pars distalis (PD) and pars intermedia (PI). Recently, we cloned three subtypes of the POMC gene in pituitary gland of barfin flounder. The present study was undertaken to elucidate whether the three POMC genes are expressed in both the PD and PI of barfin flounder pituitary, and to identify peptides derived from POMCs in these lobes. We amplified the transcripts of POMC-A, -B and -C in both the PD and PI by the reverse transcription-polymerase chain reaction. In situ hybridization also detected signals for these three subtypes in the PD and PI. These results demonstrated that all three POMC genes are expressed in both the PD and PI of barfin flounder pituitary. By mass spectrometric analyses, ACTH-A, Des-acetyl (Ac)-α-MSH-A/B (amino acid sequence of α-MSH-A is identical to that of α-MSH-B), β-MSH-A, corticotropin-like intermediate lobe peptide (CLIP)-A, and N-terminal peptide (*N*-POMC)-A were identified in the PD. Moreover, Des-Ac-α-MSH-A/B, α-MSH-A/B, β-MSH-A and -B, *N*-β-lipotropin-A, CLIP-A, *N*-Ac-β-END-A_{1-41} (C-terminally truncated form of *N*-Ac-β-END-A), and *N*-POMC-A were identified in the PI. Predominant detection of POMC-A-derived peptides indicates the greatest production of POMC-A and no detection of POMC-C-derived peptides indicates the lowest production of POMC-C in both the PD and PI. ACTH-A is specifically produced in the PD, however, the occurrence of Des-Ac-α-MSH-A, CLIP-A, and β-MSH-A shows that the entire POMC-A is further cleaved into small peptides as in the PI. In the PI, some peptides receive modification or truncation as shown by the occurrence of α-MSH-A/B and *N*-Ac-β-END-A_{1-41}. These results show differential posttranslational processing of POMC between the PD and PI in barfin flounder pituitary.

Keywords: Adrenocorticotropic hormone; Corticotropin-like intermediate lobe peptide; Endorphin; Flounder; Lipotropin; Melanophore-stimulating hormone; Pituitary; Proopiomelanocortin; Posttranslational processing

1. Introduction

Proopiomelanocortin (POMC) serves as a precursor of several hormonal peptides such as adrenocorticotropic hormone (ACTH), melanophore-stimulating hormone (MSH), lipotropin (LPH) and endorphin (END) (Takahashi and Kawauchi, 2005). Tetrapod POMC is characterized by the presence of three MSH segments and a single END segment. α-MSH is contained in the ACTH segment, β-MSH and β-END in the β-LPH segment, and γ-MSH in the pro-γ-MSH segment. The major POMC-producing tissue is the pituitary gland, in which differential posttranslational processing takes place in the pars distalis (PD) and pars intermedia (PI). The major products in the PD are ACTH, pro-γ-MSH, γ-LPH, and β-END, whereas those in the PI are α-MSH, β-MSH, γ-MSH, *N*-β-LPH, *N*-acetyl (Ac)-β-END

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with a C-terminal truncation, and corticotrophin-like intermediate lobe peptide (CLIP) (Castro and Morrison, 1997; Smith and Funder, 1988).

The structure of the teleost POMC is characterized by a consistent lack of γ-MSH segments (Takahashi and Kawauchi, 2005). Also in teleost pituitary, POMC is produced in both the PD and PI, while the population of POMC cells in the PD is remarkably smaller than that in the PI (Naito et al., 1984; Sánchez Cala et al., 2003; van Den Burg et al., 2001). This difference in the number of POMC cells correlates with the preferential isolation or detection of PI-specific POMC-derived peptides from whole pituitary of teleost (Takahashi et al., 2000, 2002; van Den Burg et al., 2001). However, neither sequencing data nor the isolation of PD-specific POMC-derived peptides such as ACTH and β-END has been reported, though these peptides have been detected by radioimmunoassay (Arends et al., 1999; Rodrigues and Sumpter, 1983; Seale et al., 2002; van Den Burg et al., 2001). Thus, the difference in posttranslational processing of POMC between the PD and PI of teleost pituitary remains to be elucidated.

The barfin flounder, *Verasper moseri*, is a large pleuronectiform fish inhabiting cold sea basins around northeast Japan. This species is promising for aquaculture and resource enhancement in northern Japan because of its high commercial value. Recently, we cloned three subtypes of POMC (POMC-A, -B, and -C) cDNA and identified several POMC-derived peptides, which could be classified as PI-specific peptides, from whole pituitary of barfin flounder (Takahashi et al., 2005). These three barfin flounder POMCs contain segments for *N*-POMC, α-MSH, β-MSH, and β-END as do other teleost POMCs, while POMC-C shows remarkable variations in the segments corresponding to *N*-POMC and β-END. Among hormonal segments of the three barfin flounder POMCs, α-MSH in POMC-A and -B is identical. More recently, POMC-producing cells were identified in the PD and PI by immunocytochemistry (Amano et al., 2005). The present study was undertaken to elucidate whether the three POMC subtype genes are expressed in both the PD and PI, and to identify PD-specific and PI-specific peptides derived from POMCs. Thus, we first examined the expression of POMC genes by reverse transcription polymerase chain reaction and in situ hybridization, and second examined the occurrence of POMC-derived peptides in barfin flounder PD and PI by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS). The obtained mass values were assigned to amino acid sequences of barfin flounder POMCs. Based on the results, the distribution and posttranslational processing of barfin flounder POMC in the PD and PI were discussed.

2. Materials and methods

2.1. Pituitary

Barfin flounder, *Verasper moseri*, was obtained from the Iwate Fisheries Technology Center, Iwate Prefecture, Japan. The fish were reared under a natural photoperiod in seawater of natural temperature. Fish were anesthetized by immersion in 0.05% of 2-phenoxyethanol for tissue sampling. For the polymerase-chain reaction, the pituitary was taken from fish weighing about 1 kg and separated into the PD and neurointermediate lobe (NIL) containing PI, and then frozen immediately in liquid nitrogen. For in situ hybridization, brains were treated as described in experiment 2.3.

2.2. Polymerase-chain reaction

Total RNA was prepared from pituitaries using Isogen (Nippon Gene, Tokyo, Japan). The template for the polymerase chain reaction (PCR) was prepared from the total RNA at 42 °C for 60 min using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The reaction mixture (50 μl) for PCR was composed of 25 μl of AmpliTaq Gold PCR Master mix (0.25 U, Applied Biosystems), 1 μl of the first strand cDNA, 1 μl of forward primer (10 μM), and 1 μl of reverse primer (10 μM). The conditions for PCR in a thermal cycler (PC-808, Astec, Fukuoka, Japan) were activation of the enzyme at 95 °C for 15 min, and 30 cycles of denaturation (60 s at 94 °C)-annealing (60 s at 57 °C)-extension (90 s at 72 °C). PCR products were 717 bp using primers 10 (5′-TGGTCAGCGGAGACGACAAC-3′) and 11 (5′-GTGAAGATGTGAACAGATTC-3′) for barfin flounder POMC-A, 662 bp using primers 12 (5′-GGCC AGAGGAGCTGTCAGT-3′) and 13 (5′-CTGCCTGTCG ACGACCTGTGTA-3′) for barfin flounder POMC-B, and 558 bp using primers 14 (5′-CATCTGCAACAACCTGA GCA-3′) and 15 (5′-TCCTTGTCTAGAATGTTCCTC-3′) for barfin flounder POMC-C.

2.3. In situ hybridization

The brains were fixed with 4% paraformaldehyde and 1% picric acid in 50 mM phosphate buffer (pH 7.3) at 4 °C for 48 h, and subsequently rinsed in cold 70% ethanol, dehydrated through graded ethanol and embedded in paraplast (Monoject, Sherwood Medical, St. Louis, MO). Serial sagittal sections were cut at 5 μm, separated into several groups, and mounted on MAS-coated slides (Matsunami, Osaka, Japan). Slides were stored at 4 °C until analysis.

After rehydration, tissue sections were preincubated with a hybridization buffer containing 20 mM Tris–HCl (pH 7.5), 1 M NaCl, 6 mM EDTA, 1× Denhardt's reagent, and 100 μg of denatured calf thymus DNA/ml at room temperature for more than 1 h in a moist chamber. The oligonucleotide probes labeled with digoxygenin (DIG) by 3′ tailing used in this study are shown in Table 1. Oligonucleotide probes were diluted in hybridization buffer to which 10% dextran sulphate had been added, and 7.5 ng of probe (85 pg/μl) was applied to each slide. As for POMC-B and POMC-C, 22 μl of each probe (85 pg/μl) was combined. Parafilm was placed over the sections, which were incubated at 45 °C overnight. After the parafilm was removed,