General and Comparative Endocrinology 176 (2012) 9–17

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

# Further evidence on acetylation-induced inhibition of the pigment-dispersing activity of α-melanocyte-stimulating hormone

Yuki Kobayashi [a,1], Kanta Mizusawa [a], Hiroaki Chiba [a], Masatomo Tagawa [b], Akiyoshi Takahashi [a,*]

[a] School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
[b] Graduate School of Agriculture, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan

ARTICLE INFO

*Article history:*
Received 13 August 2011
Revised 1 December 2011
Accepted 2 December 2011
Available online 14 December 2011

*Keywords:*
Japanese flounder
Melanocortin receptor (MCR)
α-Melanocyte-stimulating hormone (α-MSH)
Melanophores
Xanthophores

ABSTRACT

Our previous studies showed that in barfin flounder, α-melanocyte-stimulating hormone (α-MSH) stimulates pigment dispersion in xanthophores, while it shows negligible effects in melanophores. The present study was undertaken to evaluate whether these results are limited to barfin flounder by using Japanese flounder. Three subtypes of proopiomelanocortin gene encoding melanocortins (MCs) were expressed in the Japanese flounder pituitary, one of which was also expressed in the skin. Expression of melanocortin 5 receptor gene (*Mc5r*) was observed in isolated xanthophores, while that of *Mc1r* and *Mc5r* was found in melanophores. In the xanthophores of Japanese flounder skin, α-MSH as well as desacetyl (Des-Ac)-α-MSH and diacetyl (Di-Ac)-α-MSH exhibited dose-dependent pigment-dispersing activities, indicating that the signals of α-MSH-related peptides were mediated by MC5R. On the other hand, α-MSH did not stimulate pigment dispersion in melanophores, while Des-Ac-α-MSH and Di-Ac-α-MSH did, thus indicating that the expression of two different types of *Mcr* is related to the decrease in α-MSH activity. Thus, the molecular repertoire in MC system observed in Japanese flounder is similar to that in barfin flounder. Moreover, the relationship between the pigment-dispersing activities of α-MSH-related peptides and the expression of *Mcr* subtypes in xanthophores and melanophores were also similar between Japanese flounder and barfin flounder. Consequently, we hypothesize that inhibition of α-MSH activity could be due to the formation of heterodimers comprising MC1R and MC5R, often observed in G-protein-coupled receptors.



## 1. Introduction

The physiological color changes in teleosts are controlled by both the rapid sympathetic system, with noradrenalin as the neurotransmitter, and the slow endocrine system, with melanocyte-stimulating hormone (MSH) and melanin-concentrating hormone (MCH) as the representative peptide hormones [2,5]. Flatfish possess the ability to change their body color, which is known as physiological color change, and accomplish camouflage by adapting body color and hue to the background of their habitat [2,16]. In case of the barfin flounder *Verasper moseri*, which is a member of the Pleuronectiformes order inhabiting the cold sea basin areas in the northeastern part of Japan, at least two types of chromatophores—melanophores and xanthophores—are associated with color change, in which pigments with black or yellow colors migrate in concert [12,31]. Recently, we proposed that MSH constitutively stimulates pigment cells, while the activities of MCH and the sympathetic system vary in response to the changes in the background color [9,16]. This hypothesis suggests that the pigment-dispersing activity of MSH appears when the potency of MCH and the sympathetic system are attenuated.

α-MSH is a member of melanocortin (MC) peptide family and is derived from a precursor protein known as proopiomelanocortin (POMC), whose major source is the pituitary [3,25,29]. Barfin flounder possess three POMCs, one is also expressed in the skin in addition to the pituitary [12,27]. Moreover, α-MSH-related peptide is detected in skin extracts, suggesting that the control of skin pigmentation by MSHs is of twofold nature: endocrine control by the pituitary and paracrine control by the skin itself [12,31]. Five subtypes of the MC receptor (MCR) have been identified, of which MC1R is the classical α-MSH receptor [6]. We have cloned cDNA for *Mc1r*, *Mc2r*, *Mc4r*, and *Mc5r* in barfin flounder [11,13,14] and found predominant expression of *Mc1r* and *Mc5r* in their skin [14].

There are three representative forms of α-MSH-related peptides [4,26], which differ by the number of acetyl groups added to the N-terminal serine residues. α-MSH possesses one acetyl group at the *N*-position and diacetyl (Di-Ac)-α-MSH possesses two acetyl groups at the *N*-and *O*-positions, while desacetyl (Des-Ac)-α-MSH has no modifications. Recently, we reported that the pigment-dis-

* Corresponding author. Fax: +81 42 778 5010.
*E-mail address:* akiyoshi@kitasato-u.ac.jp (A. Takahashi).
[1] Present address: Graduate School of Integrated Arts and Sciences, Hiroshima University, 1-7-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-8521, Japan.

0016-6480/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2011.12.001

persing activities of α-MSH-related peptides were not proportional to the degree of acetylation in the chromatophoric skin cells of barfin flounder [12]. Specifically, α-MSH exhibited higher pigment-dispersing activities than Des-Ac-α-MSH in xanthophores. However, while Des-Ac-α-MSH stimulated pigment dispersion in melanophores, α-MSH had a negligible effect [14]. Moreover, we reported that *Mc5r* transcript, but not other *Mcr* subtypes, was detected in xanthophores, suggesting that acetylation increases the binding affinity of α-MSH to MC5R in the xanthophores. Interestingly, both *Mc1r* and *Mc5r* transcripts were detected in melanophores; hence, we hypothesized that a heteromer consisting of MC1R and MC5R may have low binding affinity for α-MSH on the basis of a growing body of evidence indicating that many G-protein-coupled receptors (GPCRs) form heterodimers that may affect ligand affinity [22].

The present study was undertaken to determine whether the relationship between the effects of α-MSH-related peptides and the expression of *Mcrs* in the chromatophores as observed in barfin flounder are restricted to this species or are common to other species by using Japanese flounder, another member of Pleuronectiformes. In other words, to support our hypothesis, we investigated whether inhibition of the pigment-dispersing activities of α-MSH is always related to the expression of two different types of *Mcrs*.

## 2. Materials and methods

### 2.1. Fish

The Japanese flounder *Paralichthys olivaceus* was purchased from Iwate Cultivating Fishery Association (Iwate, Japan). All experiments were conducted in accordance with the Kitasato University guidelines for the care and use of animals. The fish were reared in indoor tanks supplied with running seawater under natural photoperiod. The total length of the fish was 27.7–37.1 cm, and the body weight (BW) was 266–611 g. For molecular cloning and expression experiments, tissue samples were collected from fish anesthetized with 0.2% 2-phenoxyethanol, and subsequently frozen in dry ice/ethanol bath. Skin samples used for measurements of pigment-dispersing activities and cell dispersion were collected from fish anesthetized with ice-cold water. Fish were used for these experiments without sex discrimination.

### 2.2. Peptides

α-MSH was purchased from the Peptide Institute (Osaka, Japan). Di-Ac-α-MSH was purchased from Sigma Chemical (St. Louis, MO, USA). Des-Ac-α-MSH was synthesized and purified according to previously described methods [28]. Barfin flounder MCH was purchased from PHJapan (Hiroshima, Japan).

### 2.3. Molecular cloning

#### 2.3.1. Nucleic acid preparation for sequence determination

Total RNA was extracted from the brain and head kidney by using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNAs were synthesized from total brain RNA for the amplification of *Mc1r*, *Mc2r*, *Mc4r*, and *Mc5r* cDNA with the SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA). First-strand cDNA was also prepared from the head kidney RNA for amplification of *Mc2r*. Custom oligonucleotides were synthesized at Nihon Gene Research Labs Inc. (Sendai, Japan).

#### 2.3.2. Amplification of DNA fragments for sequence determination

Polymerase chain reaction (PCR) using a thermal cycler (MJ Mini; BIO-RAD, Hercules, CA, USA) under conventional conditions was performed to amplify the DNA fragments with HotStar Taq Master Mix (Qiagen, Hilden, Germany) or Takara LA Taq (Takara, Otsu, Japan). Amplification procedures were identical for all four *Mcr* cDNAs. First, the middle segment of the cDNA of each *Mcr* was amplified from first-strand cDNA by PCR using HotStar Taq DNA polymerase with primers designed from fish *Mcr* nucleotide sequences. Then, the 3′ region of each *Mcr* cDNA was amplified from first-strand cDNA by 3′ rapid amplification of cDNA ends (3′RACE) using HotStar Taq DNA polymerase with gene-specific primers and the Universal Primer A Mix (UPM) provided in the SMART RACE cDNA Amplification Kit. The 5′ region of each *Mcr* cDNA was amplified from first-strand cDNA by 5′RACE using HotStar Taq DNA polymerase with UPM and gene-specific primers. Finally, each *Mcr* cDNA containing the full-length reading frame was amplified from first-strand cDNA by PCR using HotStar Taq DNA polymerase with gene-specific primers. Primer sequences are listed in Table 1.

#### 2.3.3. Sequence determination and data processing

PCR-amplified DNA was purified by agarose gel electrophoresis (NuSieve GTG Agarose; Cambrex Bio Science, Rockland, ME, USA). DNA was extracted from the agarose gel using a QIAEX II Gel Extraction Kit (Qiagen), ligated into plasmid pT7 Blue T-Vector (Novagen, Madison, WI, USA) or pSTBlue-1 AccepTor Vector (Novagen), and transfected into JM109-competent cells. Recombinant plasmid DNA was prepared using alkaline-SDS method, and both strands were sequenced using a capillary DNA sequencer (3130-Avant Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing Ready Kit ver. 3.1. DNASIS-Pro (Hitachi Software Engineering, Yokohama, Japan) was used to process nucleotide and amino acid sequences, to calculate amino acid sequence identity, to align amino acid sequences, and to construct a phylogenetic tree by using the neighbor-joining (NJ) method [19]. Transmembrane domains were predicted using a program for the prediction of transmembrane

**Table 1**
Custom oligonucleotide primers used for PCR to amplify cDNA fragments of Japanese flounder *Mc1r*, *2r*, *4r*, and *5r*.

| Primer | Target | Nucleotide sequence |
|---|---|---|
| MC1-fw-1 | MC1R | 5′-CGA CAT GCT CGT CAG TGT CA-3′ |
| MC1-rv-1 | MC1R | 5′-GAA GAA CGG GCC CCA GCA TA-3′ |
| GSP-MC1-fw-1 | MC1R | 5′-CCG TGT TGT ATC TGC ACA TG-3′ |
| GSP-MC1-rv-1 | MC1R | 5′-GAG AAG CAT GAA CAC GGT CT-3′ |
| GSP-MC1-fw-2 | MC2R | 5′-CTC ACG CCA GAC AGT CAT TA-3′ |
| GSP-MC1-rv-2 | MC2R | 5′-TGC ATG CCT GCA GGT CGA CT-3′ |
| MC2-fw-1 | MC2R | 5′-CCC CGT CCC TCT CTT CTT CA-3′ |
| MC2-rv-1 | MC2R | 5′-TTT AGG GCA TGG ATG CGA GC-3′ |
| MC2-fw-2 | MC2R | 5′-GGT GTG TCA GCC ATA CTT AC-3′ |
| MC2-rv-2 | MC2R | 5′-CTC GAA CAG CGA TCG GTG GC-3′ |
| GSP-MC2-fw-1 | MC2R | 5′-TCC GTT TTT TGT CCA CCT TA-3′ |
| GSP-MC2-rv-1 | MC2R | 5′-GAG TGA AGG TCC CTA TTT CG-3′ |
| GSP-MC2-fw-2 | MC2R | 5′-TCA GCA AGT GGC TGG GAA GA-3′ |
| GSP-MC2-rv-2 | MC2R | 5′-CAT GTT TTC TTC TGT GGA TA-3′ |
| MC4-fw-1 | MC4R | 5′-GAT GTT ACG AGC AGC TGC TG-3′ |
| MC4-rv-1 | MC4R | 5′-TGA GAG CAG CAG AAA ATC TC-3′ |
| GSP-MC4-fw-1 | MC4R | 5′-TCA TCA TGT GCA ACT CCG TC-3′ |
| GSP-MC4-rv-1 | MC4R | 5′-TGT TCT CCA ACA GGC TGA CG-3′ |
| GSP-MC4-fw-2 | MC4R | 5′-GTC CTC ACA ATG AGG ACG AT-3′ |
| GSP-MC4-rv-2 | MC4R | 5′-TGT TCC TTG GTT GAA TCC TG-3′ |
| MC5-fw-1 | MC5R | 5′-CTG CAG TTT GGC AGT AGC AG-3′ |
| MC5-rv-1 | MC5R | 5′-CAT GCG TTC CTC AGG CTG CA-3′ |
| GSP-MC5-fw-1 | MC5R | 5′-TGT CCA TCT GCC GGA TGA AG-3′ |
| GSP-MC5-rv-1 | MC5R | 5′-CAT CCT GTT GGG GAT CTT CA-3′ |
| GSP-MC5-fw-2 | MC5R | 5′-CAT CTG AGC CAC TTG TGG AA-3′ |
| GSP-MC5-rv-2 | MC5R | 5′-AGT CAG TCC ATC CTA CCC AT-3′ |
| UPM | MC1R, 2R, 4R, 5R | 5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3′ |

Synthesis of primers was performed by Nihon Gene Research Lab. (Sendai, Japan).