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cDNA cloning of proopiomelanocortin (POMC) and mass spectrometric identification of POMC-derived peptides from snake and alligator pituitaries

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Abstract

Proopiomelanocortin (POMC) is the precursor of melanocyte-stimulating hormone (MSH) and β-endorphin, and is suggested to have evolved by the insertion and deletion of ancestral MSH segments. Here, the primary structure of POMC was determined with cDNA cloning of brown tree snakes of Squamata and American alligators of Crocodylia to show an overview of the molecular evolution of POMC in reptiles. Snake and alligator POMCs are composed of α-, β-, and γ-MSH segments and a single β-END segment as in other tetrapods; however, the γ-MSH segment in snake POMC has a mutation in the essential sequence from His-Phe-Arg-Trp to His-(d)-(d)-Arg, in which (d) means deletion. It is conceivable that the ancestry of snake γ-MSH had weak functional constraint and lacked biological significance during evolution. Phylogenetic analyses using the neighbor-joining method show that snake prePOMC is most diverged, and alligator prePOMC is most conserved in reptilian POMCs while it shows the highest sequence identity with ostrich prePOMC. These relationships are comparable to those observed in mitochondrial DNA. On the other hand, analyses of the pituitary with mass spectrometry revealed several peptides by post-translational processing as predicted by the locations of processing sites consisting of basic amino acid residues in snake and alligator POMCs. Remarkably, the monobasic site at the N-terminal side of the snake β-MSH is suggested to act as a processing site. Thus, the study shows the divergence of snake POMC such as the critical mutation of γ-MSH and high conservation of hormone organization of alligator POMC.
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Keywords: Alligator; Molecular evolution; Primary structure; Proopiomelanocortin; Reptile; Snake

1. Introduction

Proopiomelanocortin (POMC) is the precursor of melanocortin (MC) peptides such as melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH), which contains an essential sequence, His-Phe-Arg-Trp, and a single β-endorphin (β-END). The number of MSH in POMC varies depending on the taxonomic group (Takahashi and Kawauchi, 2006a,b). POMCs of lobe-finned fish and primitive ray-finned fish have three MSH segments, namely α-, β-, and γ-MSH, while more derived ray-finned fish POMC lacks γ-MSH, and cartilaginous fish POMC has four MSHs. Moreover, agnathan lampreys have two different forms of POMCs containing one or two MSHs (Takahashi et al., 1995). Thus, POMC is thought to have diverged by the insertion and deletion of an MSH segment during the early evolution of vertebrates (Takahashi and Kawauchi, 2006a,b). In contrast, POMCs of tetrapods including amphibians, reptiles, birds, and mammals consistently possess α-, β-, and γ-MSH as in lobe-finned fish and primitive ray-finned fish (Takahashi

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and Kawauchi, 2006a,b). While some fish POMCs have mutations in the essential sequence of γ-MSH (Alrubaian et al., 1999; Amemiya et al., 1997; Danielson et al., 1999; Dores et al., 1997, 1999), comparable mutations have not been observed in tetrapod γ-MSH.

MCs and β-END are usually flanked by sets of paired basic amino acid residues on POMC. After biosynthesis, POMC undergoes cleavage by prohormone convertase, such as PC1 and PC2, at basic residues, which generates a variety of peptides (Benjannet et al., 1991; Castro and Morrison, 1997; Smith and Funder, 1988). POMC is mainly produced in the pars distalis and pars intermedia of pituitary glands, in which tissue-specific cleavage and modification take place; ACTH and β-END are preferentially generated in the pars distalis, and MSHs and N-terminally acetylated β-END in the pars intermedia (Castro and Morrison, 1997; Smith and Funder, 1988; Takahashi and Kawauchi, 2006a). However, the N-terminal side of the β-MSH segment is flanked by a single basic amino acid residue in rodents (Drouin and Goodman,1980; Uhler and Herbert, 1983) and birds (Naudé et al., 2006; Takeuchi et al., 1999). Moreover, processing sites at N-terminal and/or C-terminal sides of the γ-MSH segment in primitive ray-finned fish such as paddlefish (Danielson et al., 1999), sturgeon (Amemiya et al., 1997), and bichir (Bagrosky et al., 2003) are incomplete, so that β-MSH or γ-MSH would not be liberated from POMC in these species.

Extant reptiles, class Reptilia, are composed of four orders including Chelonia (tortoises and turtles), Sphenodonta (tuatara), Squamata (lizards and snakes), and Crocodylia (crocodiles). In this group, the organization of peptide hormones within the POMC has been determined in mud turtles of Cryptodira, one of the two suborders of Chelonia (Shen et al., 2003), and leopard geckos of Lacertilia (Endo and Park, 2004), one of the two suborders of Squamata. These reports have shown that all MCs and β-END are flanked by two or three successive basic amino acid residues as in mammalian POMCs except for leopard gecko γ-MSH, the C-terminal side of which has a single Lys residue. Thus, the overall molecular architecture of reptile POMCs is supposed to be the same as other tetrapod POMCs; however, sequence determination of POMC in taxonomic groups, in which the structure of POMC has not been elucidated, is necessary to evaluate this hypothesis. The present study was undertaken to clone POMC cDNA from brown tree snakes of Serpentes, the other suborder of Squamata, and American alligators of Crocodylia to show an overview of the molecular evolution of POMC in reptiles.

2. Materials and methods

2.1. Pituitary

Brown tree snakes (*Boiga irregularis*) were collected on the island of Guam by personnel of the US Department of Agriculture as part of the tree snake eradication program. After capture they were placed in cloth bags then killed by decapitation. Pituitaries were collected under field conditions without adequate light or a microscope. It was not possible to separate different regions and it was necessary to freeze the tissue as soon as possible (ambient temperature was ~35 °C). Alligator tissues were collected from adult American alligators (*Alligator mississippiensis*) killed by hunters during the annual sanctioned hunt in Louisiana. Alligators were shot in the spine, the head removed by biologists from the Louisiana Department of Wildlife and Fisheries, and then brought to a workshop at the Rockefeller Wildlife Refuge where they were dissected. Again, it was not possible to divide the pituitaries into regions because of ambient temperature and the need to freeze the tissue as soon as possible. Pituitaries were frozen on dry ice and stored at −80 °C.

2.2. Nucleic acid preparation and polymerase chain reaction

The synthesis of oligonucleotides was performed by Nihon Gene Research Labs Inc. (Sendai, Japan). Total RNA was prepared from pituitaries using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNA was prepared from total RNA at 37 °C for 60 min using Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany). 5′RACE and 3′RACE were performed using a SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA). Polymerase chain reaction (PCR) was performed using a HotStarTaq Mater Mix Kit (Qiagen) in a thermal cycler (PC-808, Astec, Fukuoka, Japan). The reaction mixture (50 μl) for PCR was typically composed of 25 μl of HotStarTaq Master Mix (2.5 U), 1 μl of template cDNA, 2 μl of forward primer (10 μM), and 2 μl of reverse primer (10 μM). When UPM in the SMART RACE cDNA Amplification Kit was used, its final concentration was 1 μM. The typical condition for PCR was activation of the enzyme at 95 °C for 15 min, and 30 cycles of denaturation (60 s at 94 °C), annealing (60 s at 45 or 55 °C), and extension (60 s at 72 °C), followed by final extension for 10 min for 72 °C.

The middle part of snake POMC cDNA was amplified from first-strand cDNA prepared from the brown tree snake pituitary using primers Bi-1 (5′-CCA TTT CCG CTG GAA CAA GT-3′) and Bi-2 (5′-ATG GC(AG) TT(CT) TT(AG) AA(GAC) AGA GT CA-3′) prepared based on the nucleotide sequence of mud turtle (Shen et al., 2003), leopard gecko (Endo and Park, 2004) and chicken POMCs (Takeuchi et al., 1999). Using gene-specific primers Bi-3 (5′-TGC CCA AGA AGA AGC GCT AC-3′) and Bi-4 (5′-ATC TGC ACT GGA GTC ACT GG-3′) based on the middle part of cDNA, the 5′ part and 3′ part of snake POMC cDNA were amplified with 5′RACE and 3′RACE, respectively. The full-length open-reading frame of snake POMC cDNA was amplified using gene-specific primers Bi-5 (5′-GCA GAT GTT AAG AGG AAG AAA GG-3′) and Bi-6 (5′-CTT TCA TTT ACA GTA GTG TAC AGC-3′). The number of clones sequenced was five for the 5′ part fragment, eight for the middle fragment, five for the 3′ part fragment, and three for the full-length.

The 3′-terminal part of alligator POMC cDNA was amplified from first-strand cDNA prepared from American alligator pituitary using degenerated primer Am-1 (5′-ATC (CA)G(GC) AAG TA(CT) GT(CG) ATG AG-3′) prepared based on the nucleotide sequence of mud turtle (Shen et al., 2003), leopard gecko (Endo and Park, 2004), chicken (Takeuchi et al., 1999), and btsPOMC cDNAs. Using gene-specific primer Am-2 (5′-CTG CTG TTC CTC CTG CCG AA-3′) based on 3′ part cDNA, 5′ part cDNA was amplified with 5′RACE. The full-length open-reading frame of alligator POMC cDNA was amplified using gene-specific primers Am-3 (5′-ACA GAC ACT CAC AGG GAG AG-3′) and Am-4 (5′-CAT CTT GTA ATT AGT GGA ACC C-3′). The number of clones sequenced was four for the 5′ part fragment, seven for the 3′ part fragment, and three for the full-length.

2.3. Nucleotide sequence analysis

The PCR-amplified cDNA product was electrophoresed on agarose gel (NuSieve GTG Agarose, Cambrex Bio Science, Rockland, ME, USA). The cDNA was extracted and purified from agarose gel using a QIAEX II Gel Extraction Kit (Qiagen), ligated into pT7 Blue T-Vector (Novagen, Madison, WI, USA), and introduced into JM109 competent cells. Recombinant plasmid DNA was prepared by the alkaline-SDS method and sequenced on both strands with a capillary DNA sequencer (3100-Avant Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Ready Kit ver. 2.0

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