General and Comparative Endocrinology 165 (2010) 163–169

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Molecular and pharmacological characterization of the melanocortin type 1 receptor in the sea bass

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ARTICLE INFO

Article history:
Received 14 May 2009
Revised 4 June 2009
Accepted 12 June 2009
Available online 17 June 2009

Keywords:
Melanocortin receptor 1 (MC1R)
Agouti-related protein (AGRP)
Constitutive activity
Inverse agonism
IBMX
Phosphodiesterase inhibitor
Fish

ABSTRACT

Melanocortin 1 receptor (MC1R) plays a key role in the physiology of the vertebrate pigment system. Point mutations producing hyperactive or inactive receptors result in darkening or paling effects, respectively. We report the molecular and pharmacological characterization, as well as the tissue expression pattern, of the sea bass Mc1r. Similar to other MC1Rs, the sea bass gene is highly polymorphic and nine DNA polymorphisms, seven of them involving an amino acid substitution, were detected. SbMc1r is mainly expressed in the testis, fat and liver with moderate levels in the ventral and dorsal skin. The sea bass receptor was activated by all the melanocortins tested, with ACTH showing the lowest efficiency. The acetylation level of the MSH isoforms seems to be critical for the effectively of the agonist. Agouti-related protein (AGRP) drastically inhibited the basal activity of the receptor *in vitro*, as an inverse agonist does, but only in the presence of phosphodiesterase inhibitors. This observation suggests that sbMc1r is constitutively activated and inversely regulated by AGRP, which is expressed in the skin of different fish species.

1. Introduction

Melanocortins are produced by posttranscriptional processing of proopiomelanocortin precursor (POMC) and comprise melanocyte-stimulating hormones (α-, β- and γ-MSH) and adrenocorticotropic hormone (ACTH; Castro and Morrison, 1997). Melanocortin signalling is mediated by binding to a family of specific G-protein-coupled receptors (GPCR) that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R–MC5R). The number of receptors diverges in teleost fish. Zebrafish (*Danio rerio*) has six MCRs, with two copies of the MC5R, while pufferfish (*Takifugu rubripes and Tetraodon nigroviris*) have only four receptors with no MC3R and only one copy of MC5R (Logan et al., 2003). In mammalian systems, MC2R is specific for ACTH. The four other MCRs distinctively bind MSHs with MC1R exhibiting the highest affinity to α-MSH (reviewed by Schiöth et al., 2005). The interaction α-MSH/MC1R plays a key point in the control of mammalian pigmentation (reviewed by Cone, 2006). MC1R is expressed by melanocytes and its activation increases intracellular cAMP levels. Such increases are responsible for the main melanogenic actions of α-MSH, including the transcriptional activation of tyrosinase (TYR), the rate limiting enzyme in melanin synthesis, which results in a switch from the production of phaeomelanin (yellow/red pigment) to eumelanin (black/brown pigment, reviewed by García-Borrón et al., 2005). Point mutations producing hyperactive or inactive receptors result in darkening (melanization) or paling (pheomelanization) effects, respectively (Robbins et al., 1993).

Melanocortin signalling is also regulated by the binding of naturally occurring antagonists, agouti and agouti-related protein (AGRP). Agouti protein is a potent melanocortin antagonist of MC1R and MC4R. In mice, agouti is mainly produced by hair follicles and locally regulates the switch between production of eumelanin and phaeomelanin by antagonizing α-MSH effects on MC1R in the follicle melanocytes. In contrast, AGRP is mainly produced within the hypothalamic arcuate nucleus and the adrenal gland and it strongly inhibits melanocortin signalling at Mc3r and Mc4r, but is not active at MC1R (Cone, 2006). In addition, agouti has been also demonstrated to inhibit the differentiation and proliferation of melanoblasts (Aberdam et al., 1998; Sviderskaya et al., 2001). The unusual allele of the agouti locus, *Ay*, is characterized by yellow fur and ubiquitous expression of agouti gene, resulting in hyperphagia, hyperinsulinemia, increased linear growth, increased propensity for developing tumors, premature infertility and maturity-onset obesity (Michaud et al., 1993; Miller et al., 1993). This metabolic syndrome is mediated by antagonizing α-MSH signalling at central MC4R (Cone, 2006).

Similar to mammalian systems, recent experiments have demonstrated the involvement of Mc1r in the colour pattern of fish.

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doi:10.1016/j.ygcen.2009.06.008

The brown mutation, observed in the cave tetra (*Astyanax mexicanus*), results in a reduced melanin content, decreased melanophore number and brownish eyes. The brown phenotype is produced by loss of function mutations in the Mc1r (Gross et al., 2009). Agouti also works as a competitive antagonist at Mc1r–Mc4r and inhibits α-MSH-induced pigment dispersion in scale melanocytes. The protein is mainly produced in the ventral skin, and inhibits Mc1r-mediated melanogenesis. It has been hypothesized that agouti acts as a melanization inhibition factor in the establishment of the dorsal–ventral colour pattering in fish (Cerdá-Reverter et al., 2005). AGRP is expressed within the fish hypothalamus and it is upregulated by fasting. However, it is also expressed in several peripheral tissues including skin. Therefore, AGRP was suggested to play a paracrine or autocrine role in the regulation of pigment pattern in fish (Cerdá-Reverter and Peter, 2003). In spite of the importance of the melanocortin system in colour development in fish, few reports have focused on the molecular characterization of the fish Mc1r (Logan et al., 2003; van der Salm et al., 2005; Selz et al., 2007) and only two reports have attempted a pharmacological characterization (Klovins et al., 2004; Haitina et al., 2007a). In this paper, we report the molecular and pharmacological characterization of the sea bass Mc1r (sbMc1r). The results provide the first evidence of constitutive activity in fish Mc1r as well as the first evidence of the participation of inverse agonists in the regulation of fish Mc1r activity.

2. Materials and methods

2.1. Molecular cloning of sea bass Mc1r

Genomic DNA isolated from blood was used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and degenerate primers designed against conserved regions of the known MCR sequences. The following reaction conditions applied: 0.2 mM dNTP, 0.4 μM FwFish and RevFish primers, 1× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. The 5′ primer (FWMC2) was a 20-mer with the sequence: 5′-CCYATGTACTGCTTYATC-3′. The 3′ primer (RevFish) had the sequence 5′-TSAGVGTGATGGCKCCCTT-3′ (Fig. 1). PCR products of about 550 base pair (bp) were isolated from low melting point (LMP) Nusieve GTG agarose gel (FMC) ligated into pGEM-T easy vector (Promega) and subsequently transformed into XLI-Blue *Escherichia coli*. One clone that contained an insert of expected size was sequenced. The 3′ and 5′ regions were resolved using the Genome Walker kit (Clontech) and following manufacturer's instruction. Specific primers for genome walking were sbMc1r_5Walker_1 (5′-ATGGCTTCCACCACGTTACTGACGCTG-3′) and sbMc1r_3Walker_1 (TTAACGCCGTGCTGTACCTGCACATGTT) primers for the first PCR in the 5′ and 3′ direction, respectively, and sbMc1r_5Walker_2 primer (5′-ATGTCGGACACGGCCAGACAG-CAGATA-3′) and sbMc1r_3Walker_2 (ATCCACGAGCATGAAAGGGG-CGATTACC-3′) and for the nested PCR, respectively (Fig. 1). Fragments were subcloned into pGEM-T easy vector and sequenced. Finally, the full coding region was amplified by PCR, using genomic DNA as template and the primers Hind-MC1R-Forward (5′-AA-TAAGCTTATGGAAATGAGCAACAGGTCCCT-3′) and XhoI-MC1R-Reverse (5′-TTACTCGAGGCCGACATTGTTGATAACCTGA-3′, Fig. 1). A 978-bp DNA fragment was subcloned into pGEM-T easy vector and sequenced twice on both strands. The nucleotide sequence of sbMc1r has been deposited with EMBL Nucleotide Sequence Database under Accession No. FN377856.

2.2. RT-PCR and Southern blot analysis

Total RNA was purified from fresh tissues (testis, ovary, intestine, fat, liver, white and red muscle, spleen, head kidney, posterior kidney, gill, dorsal skin, ventral skin, eye, heart, pituitary and brain) and treated with RQ1-DNAse (Promega). Superscritp II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming with oligo $(dT)_{12-18}$ (Invitrogen). The cDNA was subsequently used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and specific primers. The 5′ primer was sbMc1r_3R2 (5′-TGTACCATACCGACAACG-3′) and the 3′ primer (MC1Rreverse) had the sequence 5′-CAGCAAAGAATGAAGACC-3′ (Fig. 1). Subsequently, PCR fragments were separated onto 1.2% agarose gel and transferred by capillarity to Hybond-N nylon membrane (Amersham). Membranes were prehybridized for at least 3 h in hybridization solution (50% formamide, 6× SSPE, 0.5% SDS, 5× Denhardt's solution and 10 mg/ml yeast tRNA type III, 1× SSPE containing 150 mM NaCl, 1 mM EDTA, 9 mM NaH_2PO_4, pH 7.4). Hybridization was carried out overnight in fresh hybridization solution containing 0.5×10^6 cpm/ml dCTP [α-^{32}P] at 42 °C. A 546-bp probe containing the central region of the sbMc1r coding region was used. Final washes were performed in 0.1× SSPE at 65 °C. After 2 h and 3 days of exposure at −80 °C, films were developed and scanned. As internal control of the reverse transcription step, touchdown PCR for 18S RNA was carried out. Primer sequences were 18S_Forw 5′-GCATGCCGGAGTCTCGTT-3′ and 5′ 18S_Rev 5′-TGCATGGCCGTTCTTAGTTG-3′.

2.3. Cell culture and transfection

If not specifically indicated, HEK cells were transfected using a modified calcium phosphate transfection method (20) and grown in DMEM (Invitrogen) containing 10% foetal bovine serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Galactosidase enzyme assay

Galactosidase enzyme assays were performed as previously described (21). Briefly the medium was removed and 50 μl of lysis buffer containing 250 mM Tris–HCl pH 8 and 0.1% Triton X-100 were added. After one round of freezing (−80 °C) and thawing, ten microlitres of the lysate were preserved for protein assays. Forty microlitres of phosphate saline buffer containing 0.5% BSA and 60 μl of substrate buffer (1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol and 200 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, ONPG) were added to the remaining lysate volume. The plate was incubated at 37 °C for 5 h and the absorbance was read at 405 nm in a 96-well plate reader (Tecan). Measurements were normalized for the protein content, determined using the BCA protein assay kit (Pierce).

2.5. Pharmacological experiments

A HEK-293 cell clone, stably expressing β-galactosidase under the control of vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP responsive elements (CRE, 21), was generated by co-transfection (50:1) of pCRE/β-galactosidase plasmid (kindly supplied by Dr. R. Cone, Vanderbilt University Medical Center) and the tgCMV/HyTK plasmid, which harbours a hygromycin resistance gene (65). Cells were selected in medium containing 400 μg/ml of hygromycin B (Invitrogen). β-Galactosidase activity was tested after incubating resistant clones in 96-well plates (15,000 cells/well) with assay medium (DMEM medium + 0.1 mg/ml bovine serum albumin, BSA + 0.1 mM isobutylmethylxanthine, IBMX) containing 10^{-6} M forskolin for 6 h. The clone showing highest response to forskolin (Clon-Q) was selected for subsequent experiments. The full coding region of the sbMc1r was released from pGEM-T easy vector (see above) and subcloned into pcDNA3 (Invitrogen). Double stable clones expressing β-galac-